

Hellmut Jork, Werner Funk,
Walter Fischer, Hans Wimmer

Thin-Layer Chromatography

Reagents and Detection Methods

Volume 1

Physical and Chemical Detection Methods
(in several parts, parts 1b and 1c in preparation)

Volume 2

Radiometric Detection Methods
(in preparation)

Volume 3

Biochemical and Biological Detection Methods
(in preparation)

Hellmut Jork, Werner Funk,
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Thin-Layer Chromatography

Reagents and Detection Methods

Volume 1a

Physical and Chemical Detection Methods:
Fundamentals, Reagents I

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Foreword

Thin-layer chromatography as practiced today seems to exist in two forms. Some scientists consider TLC to be a qualitative separation tool for simple mixtures where speed, low cost, and simplicity are its virtues. Others regard TLC as a powerful separation tool for the quantitative analysis of complex mixtures with a high sample throughput because of parallel sample processing, and as a technique that can tolerate cruder samples than column methods because the stationary phase is disposable, and which provides flexibility in the method and choice of detection since at the time of detection the separation is static and the layer open to inspection. Both groups of scientists use the same approaches and employ the same physical principles to achieve a separation, but only the second group does so in an optimized way. There remains an information gap which good books can fill to re-educate the scientific community of the current standing of TLC. I am delighted to affirm that the present book takes a needed step in this direction. As more scientists become acquainted with the modern practice of TLC they will need reliable and unbiased sources of information on the myriad of factors that influence quantitation of TLC chromatograms to avoid common pitfalls that follow in the wake of any technological advance. From physical principles, to working instruments, to the methodological requirements of an analytical protocol the reader will find such information, and just as importantly, experience, distilled into this book.

It seems to be a fact of life that real samples are too dilute or too complex for direct analyses no matter what new technology is available simply because the demand for analytical information is being continuously raised to a higher level. At this interface chemical intuition has always played an important role. Selective chemical reactions provide the methods to manipulate a sample to reveal the information desired. They provide the means to increase the response of an analyte to a particular detector and increase the selectivity of an analysis by targeting certain components of the sample to respond to a selected detector. Micropreparative chemistry and TLC have a long history of association because of the convenience of these reactions when performed with a static sample and because in TLC the separation and detection processes can be treated as separate steps and optimized independently of each other. A further important character of this book is the practical way it marries chemical and instrumental principles together providing an integrated source to the most important chemical reactions available and the details of their application to particular sample types. Until new detection principles are available, these reactions represent the most practical and,

in many cases, an elegant solution to difficult analytical problems. This book should serve to revive interest in this area and to provide a methodological source for their practice.

The power of TLC is in its flexibility as a problem solving tool. As the problems in analysis become more complicated the sophistication by which we approach those problems is ever increasing. However, it behooves us as analytical chemists not to forget our fundamental training in chemistry and to apply those principles to today's problems. It is just this feature that the reader will find instilled into this book.

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Preface

This book is the result of cooperation between four colleagues, who have been working in the field of thin-layer chromatography for many years and, in particular, took an active part in the development from hand-coated TLC plates to commercially available precoated plates and instrumental thin-layer chromatography. This development was accompanied by improvements in the field of detection of the separated zones. In particular, it became necessary to be able to deal with ever decreasing quantities of substance, so that the compilation "Anfärbereagenzien" by E. Merck, that had been available as a brochure for many, many years, no longer represented the state of the art of thin-layer chromatography.

It was against this background and in view of the fact that there is at present no contemporary monograph on thin-layer chromatography that this book was produced. It is intended as an introduction to the method, a reference book, and a laboratory handbook in one, i.e., far more than just a "Reagent Book".

The first part of the book consists of a detailed treatment of the fundamentals of thin-layer chromatography, and of measurement techniques and apparatus for the qualitative and quantitative evaluation of thin-layer chromatograms. In situ prechromatographic derivatization techniques used to improve the selectivity of the separation, to increase the sensitivity of detection, and to enhance the precision of the subsequent quantitative analysis are summarized in numerous tables.

Particular attention has been devoted to the fluorescence methods, which are now of such topicality, and to methods of increasing and stabilizing the fluorescence emissions. Nowhere else in the literature is there so much detailed information to be found as in the first part of this book, whose more than 600 literature references may serve to stimulate the reader to enlarge his or her own knowledge.

Nor has a general introduction to the microchemical postchromatographic reactions been omitted; it makes up the second part of the book.

This second part with its 80 worked-through and checked detection methods forms the foundation of a collection of reagent reports (monographs), which will be extended to several volumes and which is also sure to be welcomed by workers who carry out derivatizations in the fields of electrophoresis and high-pressure liquid chromatography. Alongside details of the reagents required and their handling and storage, the individual reports also contain details about the reaction concerned.

Wherever possible, dipping reagents have been employed instead of the spray reagents that were formerly commonplace. These make it easier to avoid contami-

nating the laboratory, because the coating of the chromatogram with the reagent takes place with less environmental pollution and lower health risks; furthermore, it is more homogeneous, which results in higher precision in quantitative analyses.

It is possible that the solvents suggested will not be compatible with all the substances detectable with a particular reagent, for instance, because the chromatographically separated substances or their reaction products are too soluble. Therefore, it should be checked in each case whether it is possible to employ the conditions suggested without modification. We have done this in each report for one chosen class of substance by working through an example for ourselves and have documented the results in the "Procedure Tested"; this includes not only the exact chromatographic conditions but also details concerning quantitation and the detection limits actually found. Other observations are included as "Notes". Various types of adsorbent have been included in these investigations and their applicability is also reported. If an adsorbent is not mentioned it only means that we did not check the application of the reagent to that type of layer and not that the reagent cannot be employed on that layer.

Since, in general, the reagent report includes at least one reference covering each substance or class of substances, it is possible to use Part II of this book with its ca. 750 references as a source for TLC applications. Only rarely are earlier references (prior to 1960), which were of importance for the development of the reagent, cited here.

There is no need to emphasize that many helpful hands are required in the compilation of such a review. Our particular thanks are due to Mrs. E. Kany, Mrs. I. Klein and Mrs. S. Netz together with Dipl.-Ing. M. Heiligenthal for their conscientious execution of the practical work.

We would also like to thank the graduate and postgraduate students who helped to check the derivatization reactions and Mrs. U. Enderlein, Mrs. E. Otto, and Mrs. H. Roth, whose capable hands took care of the technical preparations for the book and the production of the manuscript. We would particularly like to thank Dr. Kalinowski (Univ. Giessen) for his magnificent help in the formulation of the reaction paths for the reagent reports. Our thanks are also due to Dr. F. Hampson and Mrs. J. A. Hampson for translating the German edition of the book into English.

We thank the Baron, J. T. Baker, Camag, Desaga, Macherey-Nagel and E. Merck companies for their generous support of the experimental work.

Our particular thanks are also due to Dr. H. F. Ebel and his colleagues at VCH Verlagsgesellschaft for the realization of our concepts and for the design and presentation of the book and for the fact that this work has appeared in such a short time.

In spite of all our care and efforts we are bound to have made mistakes. For this reason we would like to ask TLC specialists to communicate to us any errors and any suggestions they may have for improving later volumes.

Saarbrücken, Giessen and Darmstadt,
October 1989

Hellmut Jork
Werner Funk
Walter Fischer
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Part I

Methods of Detection

1 Introduction

The *separation methods* routinely employed in the laboratory include the various chromatographic and electrophoretic techniques, whose selectivity is continually being increased by the introduction of new adsorbents, e.g. with chemically modified surfaces (Fig. 1).

Detection methods, which provide real information concerning the separated substances, are necessary in order to be able to analyze the separation result and separation performance achieved by such a system.

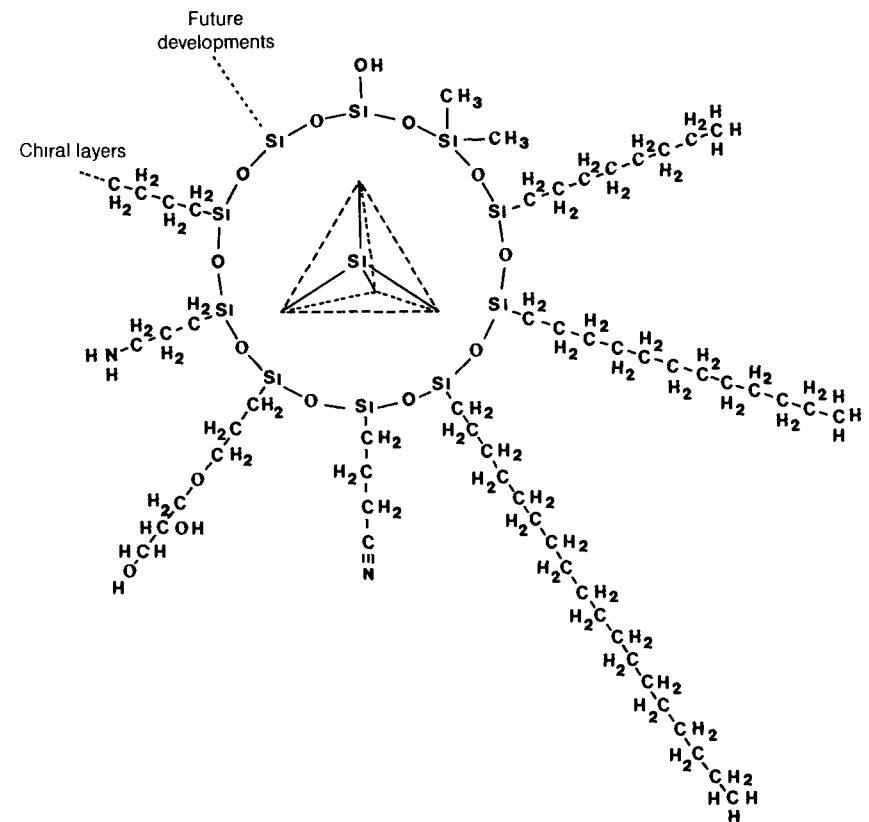


Fig. 1: Schematic view of the surface-modified silica gels at present commercially available.

In spite of numerous advances in the field of detection there are not and never have been any genuinely *substance-specific* chemical detection reactions. This means that, unlike the spectrometric methods, the methods of detection normally employed in chromatography cannot be employed for an unequivocal identification of compounds, they can only provide more or less definite indications for the characterization of the separated substances. Universal reagents are usually employed for a first analysis of the separation of samples of unknowns. This is then followed by the use of group-specific reagents. The more individual the pieces of information that can be provided from various sources for a presumed substance the more certainly is its presence indicated. However, all this evidence remains indicative; it is not a confirmation of identity.

The detection methods also serve especially to increase sensitivity and selectivity in addition to providing evidence concerning the quality of the separation. In the case of thin layer chromatography the *selectivity of the separation* which is achieved by the various techniques employed (e.g. multiple development, gradient elution, sequence TLC, AMD, HPPLC or OPLC techniques) (Fig. 2) is accompanied by *specificity of detection* [1–3]; the selectivity of detection can also be increased by the combination of several detection methods as shown in Figure 2. After chromatographic development the chromatogram may be regarded as being rather like a “diskette” with the individual pieces of information stored on it [4].

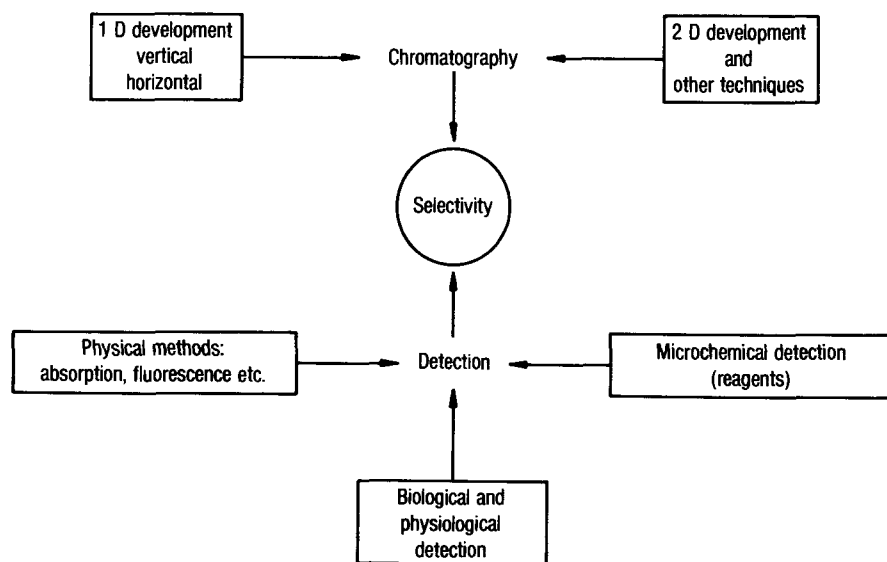


Fig. 2: Increasing the selectivity by combinations of methods.

The information can be read off as required at any time separately from the development process both in space and time. This possibility is *only* available in thin layer chromatography, because there is no direct on-line coupling between chromatographic development and detection. Thus, the analysis of a thin layer chromatogram can be repeated at any time as desired or carried out again according to other criteria than those employed in the first analysis. It follows that the detection technique does not restrict the choice of mobile phase. The chromatographic conditions can always be chosen to give the best separation for the particular sample, since the chromatogram is freed from mobile phase before detection is undertaken.

In addition to these advantages TLC also possesses other merits which ensure that it occupies a firm place in the arsenal of analytical techniques as a method for the separation of micro-, nano- and picogram quantities [5].

The method

- is easy to learn technically;
- is rapidly carried out, especially as HPTLC technique;
- is always available for use, since the precoated layers can usually be employed without pretreatment;
- can readily be monitored because the whole chromatogram (including the substances remaining at the start) can be taken in at a glance and it is not necessary to elute the individual components;
- does not require a regeneration step, since TLC and HPTLC plates are disposable items;
- can be economically employed for routine use because the consumption of mobile phase is low and, hence, there are scarcely any disposal problems (for instance up to 70 samples can be analyzed alongside each other and with authentic standard substances in a linear chamber with a very few milliliters of mobile phase [6]);
- is very adaptable: acidic, basic or purely aqueous mobile phases can be employed as can neutral lipophilic solvents, thus lending the whole thin layer chromatographic system a high degree of flexibility.

As a result of these merits thin layer chromatography finds application all over the world. The frequency of its application is documented in Figure 3. This CA search only includes those publications where TLC/HPTLC are included as key words. The actual application of the method is very much more frequent. The method is employed as a matter of course in many areas of quality control and routine monitoring of product purity. This was also true in the 1970s when the rapid development of high performance liquid chromatography (HPLC) led to a

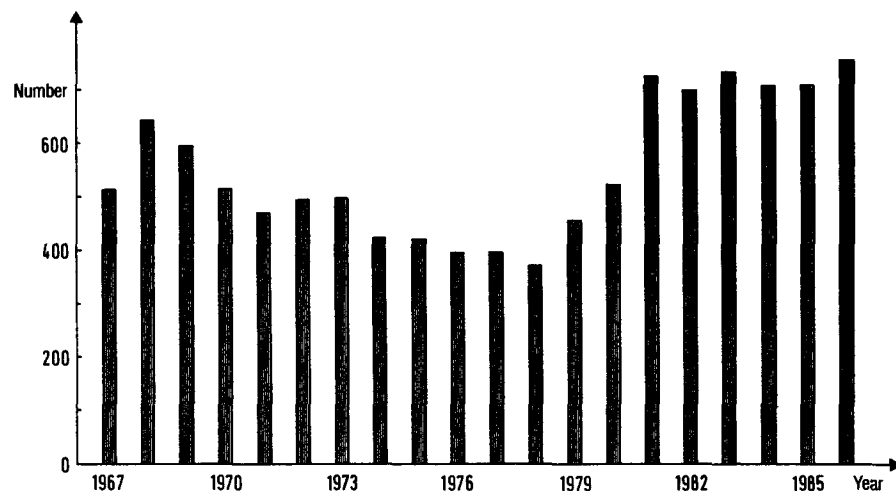


Fig. 3: Frequency distribution of TLC/HPTLC publications over the years 1967 – 1986 (search made from Chemical Abstracts).

temporary reduction in the frequency of academic TLC publications. Today both methods stand side by side as separation techniques in their own right. The analyst employs them where their respective advantages are evident. Economic considerations naturally have to be taken into account too [7] and the balance here is probably tipped in favor of thin-layer chromatography.

The information produced in a TLC separation can be recorded for storage or documentation. This can take the form of a manual-graphical reproduction, a photocopy, photograph or autoradiograph [8–10]. Increasingly, however, the chromatogram is scanned (fluorescence or absorption scanning curves) or the raw data are stored in data storage systems. The instrumentation required for these processes will be discussed in the subsequent chapters. A general discussion of documentation will follow in Chapter 4.

In principle it is possible to employ physical, microchemical and biological-physiological methods for detection in TLC (Fig. 2).

Physical methods: Physical methods include photometric absorption and fluorescence and phosphorescence inhibition, which is wrongly referred to as fluorescence quenching [1], and the detection of radioactively labelled substances by means of autoradiographic techniques, scintillation procedures or other radiometric methods. These methods are *nondestructive* (Chapt. 2).

Microchemical reactions: These can be carried out either with universal reagents [11] or with such substances which react with particular functional groups (group-characterizing reagents). If the separation process ensures that only *one* component occurs at a particular spot on the chromatogram, then this can be detected “substance-specifically”. But specificity in an unequivocal sense can only be produced by a combination of the separation and the detection process. (The same is true of other forms of detection.)

Biological-physiological detection: The methods involved here take account of the biological activity of the separated components independent of their physical or chemical properties [12].

Their use is to be recommended [13] because

- such methods are highly specific (independent of the separation process);
- ineffective accompanying substances do not interfere with the investigation so that previous clean-up can often be omitted;
- the detection limits are comparable with those of classical detection methods.

These methods are employed for the detection and determination of antibiotics and substances with similar effects, like alkaloids, insecticides, fungicides, mycotoxins, vitamins, bitter principles and saponins [14].

We intend to devote separate volumes to each method of detection in the order discussed above.

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2 Physical Methods of Detection

2.1 General

Physical detection methods are based on inclusion of substance-specific properties. The most commonly employed are the absorption or emission of electromagnetic radiation, which is detected by suitable detectors (the eye, photomultiplier). The β -radiation of radioactively labelled substances can also be detected directly. These *nondestructive* detection methods allow subsequent micropreparative manipulation of the substances concerned. They can also be followed by microchemical and/or biological-physiological detection methods.

A distinction is normally made between the visible and ultraviolet regions of the spectrum when detecting absorbing substances. Detection in the visible part of the spectrum can be carried out with the eye or with a photomultiplier.

2.2 Detection of Absorbing Substances

2.2.1 Visual Detection

The success of separation of *colored* compounds is usually monitored visually. Such compounds absorb a particular portion of the polychromatic (white) light in the visible wavelength range. The remaining radiation (complementary radiation) is reflected and detected by the eye; it determines the color of the substance zone. Table 1 correlates the wavelengths, colors and complementary colors.

Table 1. Correlation of wavelength, color and complementary color [1].

Wavelength [nm]	Color of radiation	Complementary color
620...700	red	bluish-green
590...620	orange	greenish-blue
570...590	yellow	blue
500...570	green	red/purple
450...500	blue	yellow
400...450	violet	yellowish green

Colorless substances absorb at wavelengths shorter than those of the visible range (the UV range normally amenable to analysis $\lambda = 400 \dots 200$ nm). Such compounds can be detected by the use of UV-sensitive detectors (photomultipliers, Sec. 2.2.3.1). Substances that absorb in the UV range and are stimulated to fluorescence or phosphorescence (luminescence) can be detected visually if they are irradiated with UV light.

2.2.2 Fluorescence and Phosphorescence Indicators

Fluorescent and phosphorescent substances are excited into an unstable energy state by UV light. When they return to the ground state they release a part of the energy taken up in the form of radiation. The emitted radiation is *less energetic* than the light absorbed and usually lies in the visible part of the spectrum. Since absorption (excitation) and emission obey a linear relationship over a certain range a reduction in absorption leads to a reduction in the luminescence, too.

This property can be applied to the detection of substances that absorb in the UV region: For on layers containing a fluorescent indicator or impregnated with a fluorescent substance the emission is reduced in regions where UV-active compounds partially absorb the UV light with which they are irradiated. Such substances, therefore, appear as *dark zones* on a fluorescent background (Fig. 4A).

This effect, which can also be produced if fluorescent substances are applied to the chromatogram by spraying or dipping after development, is an absorption effect and not a quenching process in the true sense of the word. It is correct to refer to fluorescence or phosphorescence diminishing. The more absorbant sample molecules there are present in the zone the darker this will appear (Fig. 4B).

This method of detection is at its most sensitive if the absorption maximum (λ_{\max}) of the sample molecule is exactly at the wavelength of the UV light employed for irradiation. The further λ_{\max} lies from this the less radiation is absorbed and the lower the sensitivity of detection. If the compound does not absorb at the wavelength of radiation or if it possesses an absorption minimum just there then such components are not detected by this method. Figure 4C illustrates this with the sweeteners saccharin and dulcin as examples.

Fluorescence and phosphorescence are both forms of *luminescence* [3]. If the emission of radiation has decayed within 10^{-8} s after the exciting radiation is cut off it is known as *fluorescence* [4], if the decay phase lasts longer (because the electrons return to the ground state from a forbidden triplet state (Fig. 5), then the phenomenon is known as *phosphorescence*. A distinction is also made between

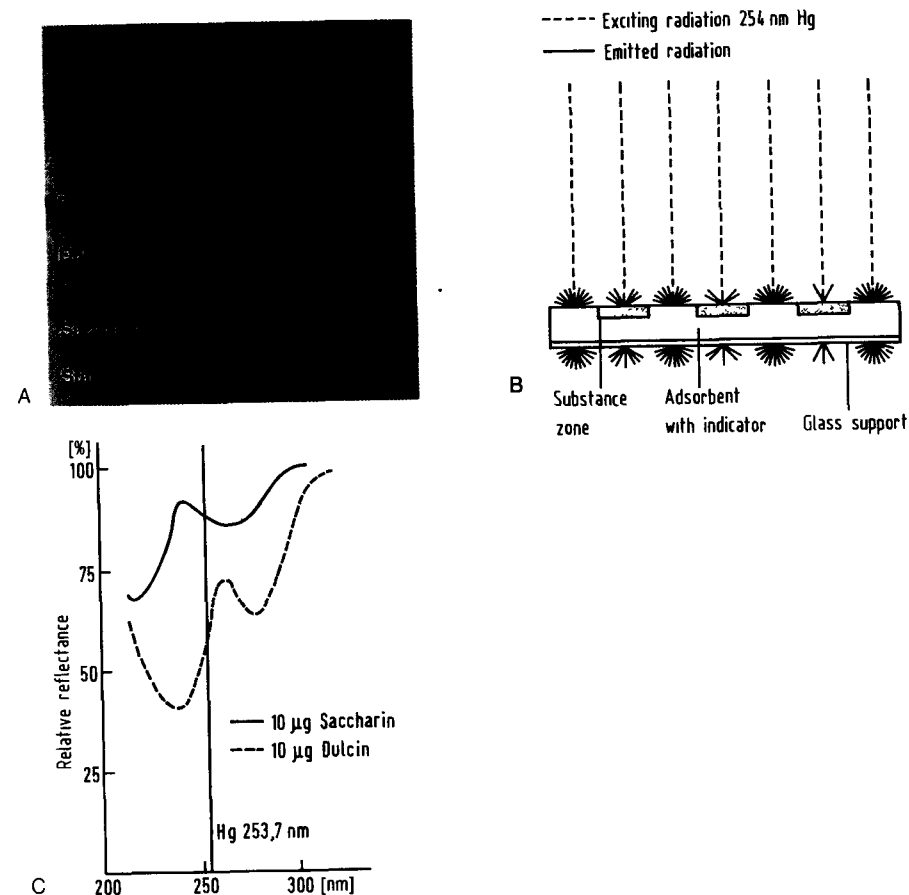


Fig. 4: Explanation of the fluorescence-quenching effect [2]. — (A) chromatograms of the same quantities of saccharin and dulcin observed under UV 254 light, (B) schematic representation of fluorescence quenching, (C) spectral reflectance curves of saccharin and dulcin.

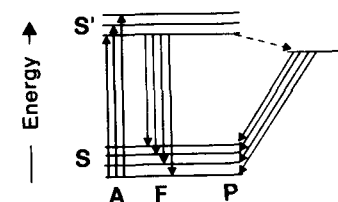


Fig. 5: Schematic representation of the electronic transitions during luminescence phenomena [5]. — A absorbed energy, F fluorescence emission, P phosphorescence, S ground state, S* excited singlet state, T "forbidden" triplet transition.

fluorescence and phosphorescence indicators. The former can be incorporated in the adsorbent layers or applied afterwards by spraying or dipping, the latter are always incorporated as homogeneously as possible into the stationary phase. Fluorescence occurs primarily in organic substances and phosphorescence, on the other hand, in inorganic compounds.

Organic fluorescence indicators for aluminium oxide, silica gel and cellulose layers (code F₃₆₆, UV₃₆₆) include:

- the sodium salt of 3-hydroxypyrene-5,8,10-trisulfonic acid [6],
- the sodium salt of 3,5-dihydroxypyrene-8,10-disulfonic acid [7],
- sodium fluorescein [8 – 11] and fluorescein [12] or 2',7'-dichlorofluorescein [13 – 19],
- rhodamine B [12, 20 – 23] and rhodamine 6G [24 – 26],
- morin [11, 24, 27 – 29],
- cyanine dyestuffs [30, 31],
- stilbene derivatives (e.g. diaminostilbenetriazine) [12, 32] and
- optical brighteners (Ultraphor WT BASF [12, 33], Calcofluor R-white [34], Leukophor).

Oxytetracycline can also be employed at low pH on calcium-containing layers [35].

The scintillators are a special type of fluorescence indicators; they are employed for the fluorimetric detection of radioactively labelled substances. They are stimulated by β -radiation to the emission of electromagnetic radiation and will be discussed in Volume 2.

The substances employed as *inorganic phosphorescence indicators* (incorrectly referred to as fluorescence indicators) include blue (tin-activated strontium compounds), yellow (uranyl acetate [36]) and yellow-green (manganese-activated zinc silicate [37] or zinc cadmium sulfide [38]) emitting substances (code F₂₅₄, UV₂₅₄). Pigment ZS-super (RIEDEL DE HAËN) has also been employed [81]. Since these are not acid-stable they are replaced by substances such as alkaline earth metal tungstates in RP phases (code F_{254s}). These possess a pale blue emission [39].

The advantages of these inorganic indicators are:

- Such indicators do not migrate during chromatography to the solvent front under the influence of either polar or nonpolar organic solvents (uranyl acetate is an exception).

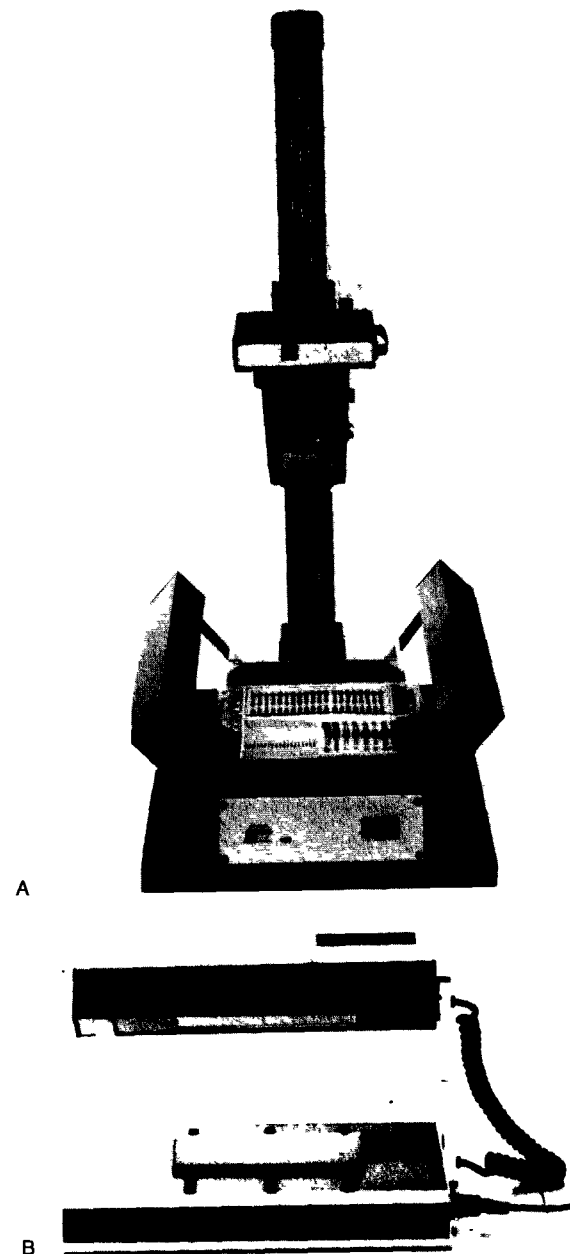
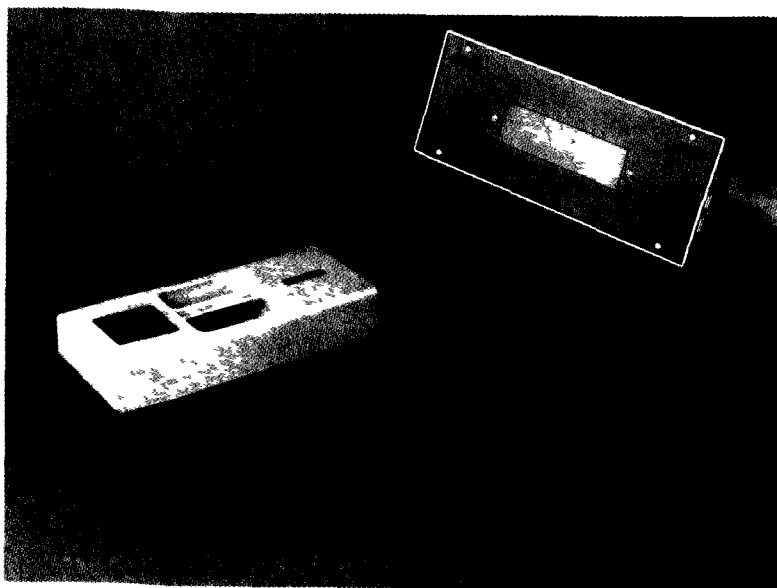


Fig. 6: UVIS and MinUVIS analysis lamps (DESAGA).



A

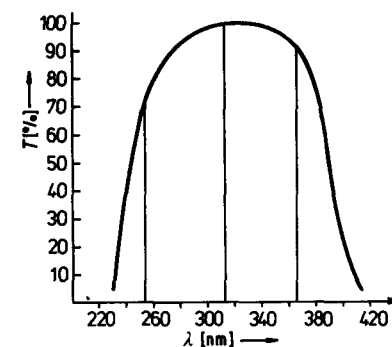


B

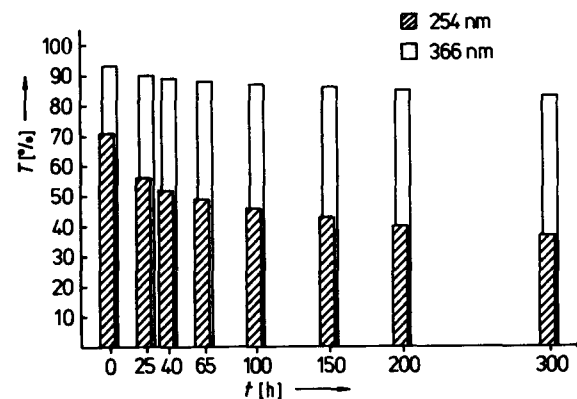
Fig. 7: UV hand lamps (CAMAG). (A) battery-powered UV lamp, (B) hand lamp with stand closed on three sides

- Short-wavelength UV radiation ($\lambda = 254 \text{ nm}$) is employed for excitation. This allows aromatic organic compounds, in particular, to be detected by fluorescence quenching. Uranylacetate may also be excited at $\lambda = 366 \text{ nm}$.
- In the most favorable cases the detection limits are from 0.1 to 0.3 μg substance per chromatogram zone.

Energetic radiation sources are required to excite phosphorescence or fluorescence. Mercury line radiators are normally employed; these are readily available as relatively cheap mercury vapor lamps. The short-wavelength line at $\lambda = 254 \text{ nm}$ is



A



B

Fig. 8: Transmittance of black light filter as a function of wavelength (A) and as a function of the length of operation at $\lambda = 254$ and 365 nm (B)

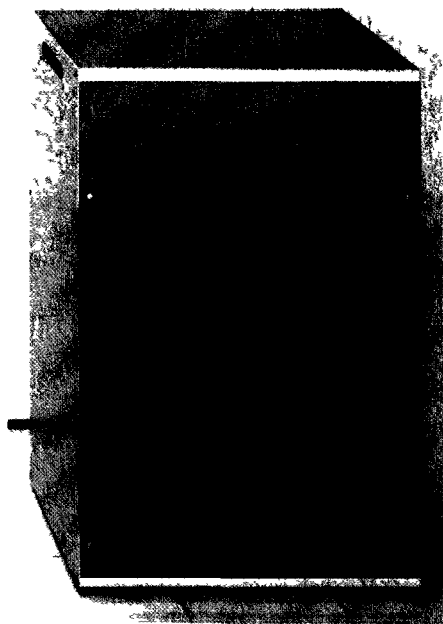


Fig. 9: HP-UVIS cabinet for UV inspection without a dark room (DESAGA).

mainly employed (although the absorption maxima of the indicators lie at $\lambda = 260$ nm or 280 nm [37]) together with the long-wavelength double line at $\lambda = 365/366$ nm. While low pressure lamps deliver short-wavelength light almost exclusively, the proportion of long-wavelength UV radiation is much higher in the case of high pressure lamps. But these have the disadvantage that they require a starting up time of 2 to 5 minutes. They also operate relatively hot and can only be re-ignited after they have cooled down.

Note: The lamp can crack if the hot bulb comes into contact with a cold TLC plate (protective housing!).

These restrictions do not apply to the less intense fluorescent tubes installed in the UVIS or MinUVIS (Fig. 6) or Universal UV lamps (Fig. 7). Black glass surrounds or screens serve as filters. Unfortunately account is often not taken of the fact that the transparency for short-wavelength UV light decreases appreciably with increasing duration of irradiation (Fig. 8). So it is advisable to change the filters of lamps intended for short-wavelength radiation at regular intervals. They can

still be employed in the long-wavelength region. This is particularly true if color photographs are taken for documentation purposes.

Combined compact instruments, where it is possible to switch from "daylight" to long- or short-wavelength UV light, are frequently offered for the examination of thin-layer chromatograms (Fig. 9). These are often fitted with a camera holder.

Caution: When working with UV light protective goggles should always be worn in order to avoid damage to the eyes.

2.2.3 Photometric Measurement of Absorption

2.2.3.1 Apparatus

Photomultipliers are appreciably more sensitive sensors than the eye in their response to line or continuum sources. Monochromators are fitted to the light beam in order to be able to operate as substance-specifically as possible [5]. Additional filter combinations (monochromatic and cut-off filters) are needed for the measurement of fluorescence. Appropriate instruments are not only suitable for the qualitative detection of separated substances (scanning absorption or fluorescence along the chromatogram) but also for characterization of the substance (recording of spectra in addition to hR_f) and for quantitative determinations.

Monochromators

Today's commercially available *chromatogram spectrometers* usually employ diffraction gratings for monochromation. These possess the following advantages over prism monochromators which are still employed in the SCHOEFFEL double-beam spectrodensitometer SD 3000 and in the ZEISS chromatogram spectrometer:

- The wavelength scale is approximately linear; this also means
- that the wavelength scan is also linear making automation easier using appropriate stepping motors;
- dispersion is almost constant and not wavelength-dependent, and
- the light transmission above $\lambda = 270$ nm is higher than is the case for prism monochromators.

However, the usable spectral region is limited by the wavelength-dependent efficiency of the gratings.

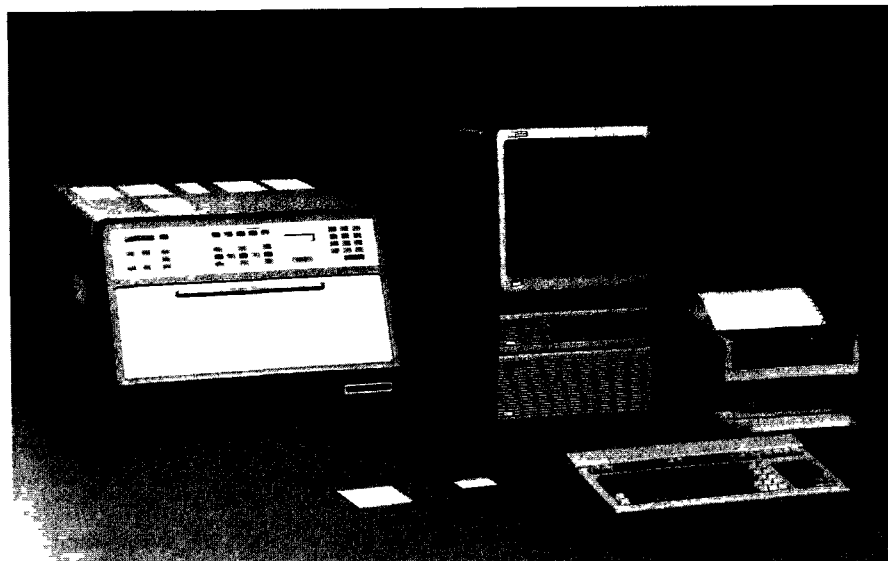


Fig. 10: TLC scanner II (CAMAG)



Fig. 11: CD-60 densitometer (DESAGA)

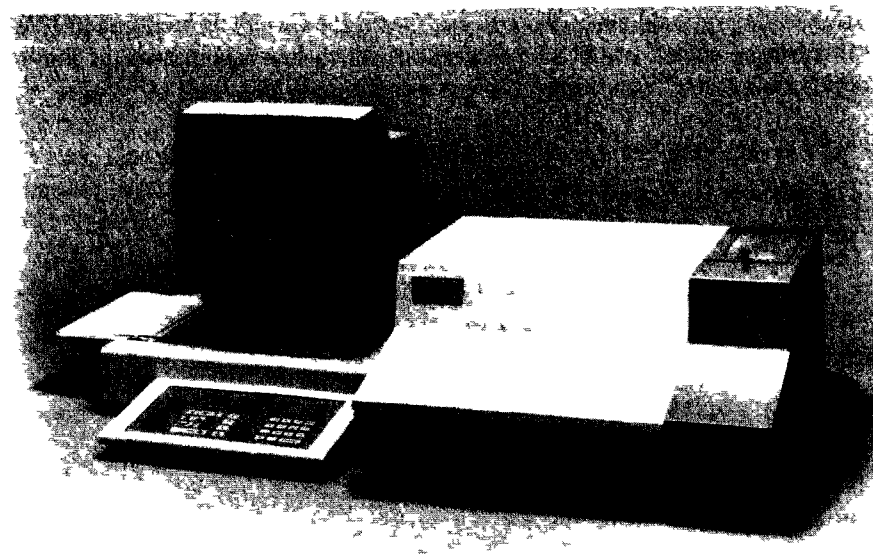


Fig. 12: Flying spot scanner CS 9000 (SHIMADZU)

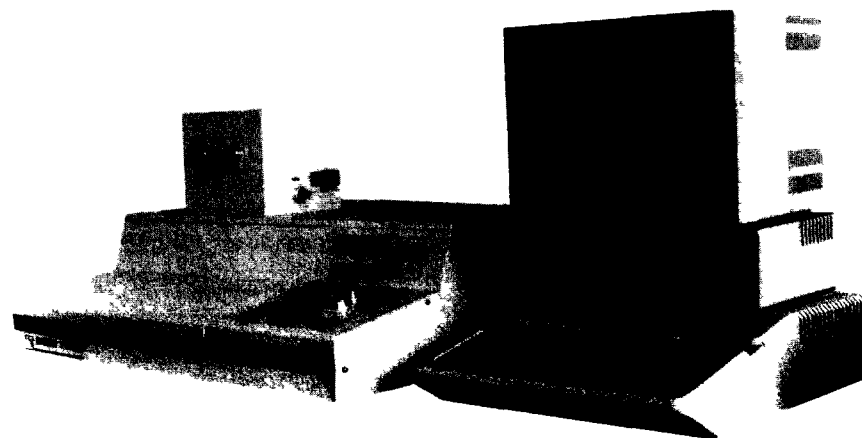


Fig. 13: FTR-20 scanner (SIGMA/BIOCHEM)

Note: Gratings should never be “polished” with the fingers or breathed on. This is also true of coated or “bloomed” gratings which have magnesium or lithium fluoride evaporated onto them.

Samples of spectrometers with grating monochromators

- TLC scanner II, CAMAG (Fig. 10)
- CD-60 densitometer, DESAGA (Fig. 11)
- CS 9000 Flying-spot scanner, SHIMADZU (Fig. 12)
- FTR-20 scanner, SIGMA/BIOCHEM (Fig. 13)

Light Sources

Lamps to be employed in photometry should

- produce radiation that is as constant as possible both in origin and intensity and
- be as good approximation as possible to a point source in order to facilitate the production of parallel beams [40].

A distinction must be made between *continuous sources* (hydrogen or deuterium lamps, incandescent tungsten lamps, high pressure xenon lamps) and *spectral line sources* (mercury lamps), which deliver spectrally purer light in the region of their emission lines.

A continuous source has to be employed to record absorption spectra. Fluorescence is usually excited with mercury vapor lamps; in the region of their major bands they radiate more powerfully than do xenon lamps (Fig. 14).

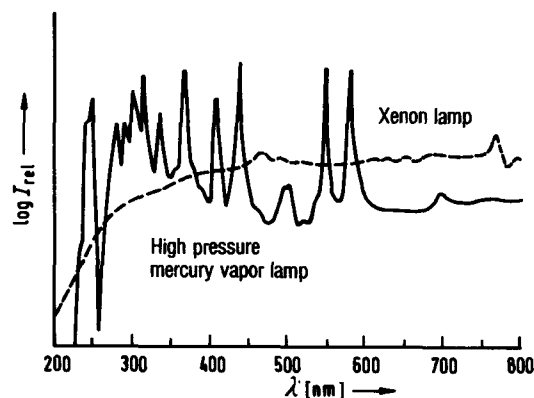


Fig. 14: Radiation characteristics of a high pressure Hg lamp (OSRAM HBO 100; continuous line) and of a xenon lamp (PEK 75; broken line) [4]. The intensity I is represented logarithmically in relative units.

Continuous sources: The sources of choice for measurements in the ultraviolet spectral region are *hydrogen or deuterium lamps* [1]. When the gas pressure is 30 to 60×10^{-3} Pa they yield a continuous emission spectrum. The maxima of their radiation emission occur at different wavelengths (H_2 : $\lambda = 280$ nm; D_2 : $\lambda = 220$ nm). This means that the deuterium lamp is superior for measurements in the lower UV region (Fig. 15).

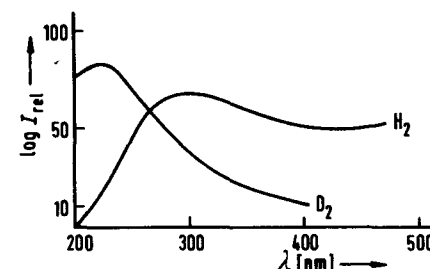


Fig. 15: Relative intensity distribution of the radiation produced by a hydrogen and a deuterium lamp.

Because of the high rate of diffusion of hydrogen the energy consumption resulting from thermal conduction is very large in the short-wave UV region and the radiation yield is relatively low. Deuterium diffuses more slowly, its thermal conductivity is lower and the radiation yield is ca. 30% higher than is the case for a hydrogen lamp.

The continuum produced by both of these lamps is accompanied by emission lines in the visible spectral region at $\lambda = 486.12$ nm (H_2) and $\lambda = 485.99$ nm (D_2); these can be employed for adjustment and calibration of the wavelength scale.

Hydrogen lamps are equipped with a rectangular slit for adjusting and centering the gas discharge; this ensures that the radiation intensity is particularly high in this region and the position of the radiation source is stable. But long-term drift cannot be excluded completely [1].

Tungsten incandescent lamps are primarily employed in the visible region ($\lambda = 320\text{--}700$ nm). They consist of an evacuated glass bulb [11] containing a thin, coiled tungsten wire which is heated to incandescence. Since tungsten melts at 3655 K the usual operating temperature is 2400 to 3450 K. The higher the temperature the higher is the vapor pressure of tungsten. The metal vapor is deposited on the relatively cool glass bulb so that the “transparency” of the glass is reduced, thus, reducing the operating life which is reported to be ca. 1000 hours at 2400 K. In order to reduce the rate of evaporation krypton or argon are often employed as protective gases, which means that 70 to 90% of the electrical energy is converted

to radiation. The fact that a considerable proportion of the energy is radiated above $\lambda = 800$ nm is a disadvantage of the tungsten incandescent lamp.

Halogen lamps are tungsten lamps whose glass bulbs also contain iodine vapor [42]. When the coil is heated incandescent volatile tungsten iodine compounds are produced in the vapor phase and these are thermally decomposed at the glowing coil. This causes a reduction in the deposition of tungsten on the surface of the glass bulb so that such lamps can be operated at higher temperatures and generate a higher light yield. Since iodine vapor absorbs UV light these lamps have a purple tinge.

High pressure xenon lamps are also employed in some TLC scanners (e.g. the scanner of SCHOEFFEL and that of FARRAND). They produce higher intensity radiation than do hydrogen or tungsten lamps. The maximum intensity of the radiation emitted lies between $\lambda = 500$ and 700 nm. In addition to the continuum there are also weak emission lines below $\lambda = 495$ nm (Fig. 14). The intensity of the radiation drops appreciably below $\lambda = 300$ nm and the emission zone, which is stable for higher wavelengths, begins to move [43].

Spectral line radiators: In contrast to the lamps described above mercury vapor lamps are gas discharge lamps [1]. They are started by applying a higher voltage than the operating voltage. The power supply has to be well stabilized in order to achieve a constant rate of radiation and the radiator must always be allowed a few minutes to stabilize after it has been switched on. The waisting of the lamp body in the middle leads to a concentration of the incandescent region. This leads to stability of the arc. Its axis remains in the same position during operation and is readily optically imaged. However, it is impossible to ensure that the arc will not "jump". In addition the relative intensities of the individual lines can change with respect to each other, which can cause a short or long-term change in the recorded baseline. The physical data concerning the most frequently employed mercury lamps are listed in Table 2.

In contrast to the *low-pressure lamps* (1–130 Pa) which primarily emit at the resonance line at $\lambda = 254$ nm, *high-pressure lamps* (10^4 – 10^6 Pa) also produce numerous bands in the UV and VIS regions (Fig. 16). Table 3 lists the emission lines and the relative spectral energies of the most important mercury lamps (see also [44]). The addition of cadmium to a mercury vapor lamp increases the number of emission lines particularly in the visible region of the spectrum [45] so that it is also possible to work at $\lambda = 326, 468, 480, 509$ and 644 nm [46].

Recently the $\text{Ar}^+/\text{He}-\text{Ne}$ lasers have been employed for the analysis of thin-layer chromatograms [259–261]. However, instruments of this type have not yet come into general use.

Table 2. Summary of the most important technical data on the most frequently employed Hg lamps [47].

Parameter	High pressure Hg lamp			
	St 41	St 43	St 46	St 48
Current type	Direct current	Alternating current	Direct current	Direct current
Supply voltage [V]	220	220	220	220
Total length [mm]	120	90		85
Usable lit length [mm]	8	20		11.5
Gas pressure [Pa]	6×10^5	0.5×10^5		6×10^5
Emitter current [A]	0.6	1.0	0.6	0.6
Emitter power [W]	45	36	33	45
Luminous intensity [cd]	95	31	70	95
Luminous density [cd/cm ²]	500	25	375	500
Examples of scanners employing them	KM-3 chromatogram spectrophotometer (C. ZEISS)	FTR-20 TLC scanner (SIGMA)	CD-60 densitometer (DESAGA)	CS-930 scanner (SHIMADZU) TLC scanner (CAMAG)

Table 3. List of emission lines and their relative intensities for the most important mercury lamps *).

Wavelength λ [nm]	Energy distribution of the emission bands		
	St 41	St 43	St 48
238 and 240	3	2	3
248	8	4	8
254	55	34	55
265	25	14	25
270	5	2	5
275	4	2	4
280	10	5	10
289	7	3	7
297	18	13	18
302	31	25	31
313	69	67	69
334	7	5	7
366	100	100	100
405 and 408	43	43	43
436	81	61	81
546	108	79	108
577 and 580	66	47	66

*) Exact intensities are given by KAASE et al. [48].

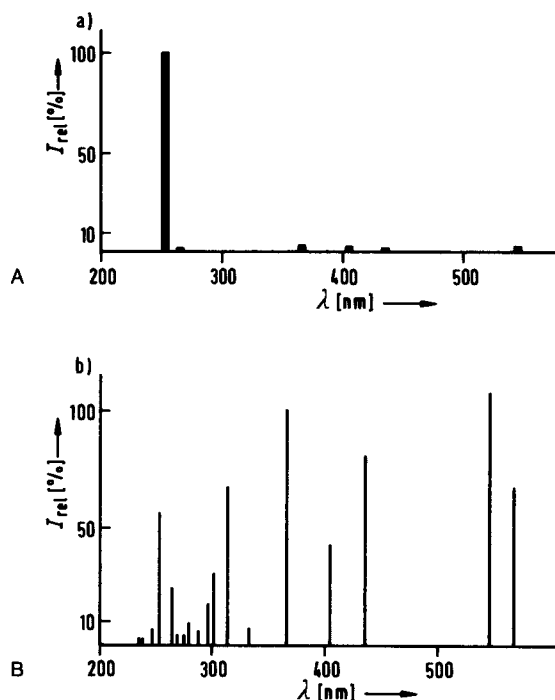


Fig. 16: Relative intensity distribution of a mercury NK 4/4 low pressure lamp (A) and of a mercury St 41 or St 48 lamp (B).

Detectors

The commercial instruments employ detectors of various types. Their utility depends fundamentally on

- the constancy with time of the photocurrent at constant radiation levels and constant external conditions,
- the proportionality of the photocurrent to the intensity of illumination and
- the signal to noise ratio of the photodetector.

A rôle is also played by the temperature and frequency dependence of the photocurrent, the variable surface sensitivity at various parts of the cathode and the vector effect of polarized radiation [40]. All the detectors discussed below are electronic components whose electrical properties vary on irradiation. The effects depend on external (photocells, photomultipliers) or internal photo effects (photoelements, photodiodes).

Photocells and photomultipliers (secondary electron multipliers, SEM) are mainly employed in photometry. These are detectors with an “external photo-effect”.

Photocells: The basic construction of a photocell is illustrated in Figure 17. A photocurrent flows when the photocathode is illuminated, this is proportional to the intensity of illumination if the supply potential has been chosen to be higher than the saturation potential. A minimal potential is required between the photocathode and the anode in order to be able to “collect” the electrons that are emitted. The sensitivity is independent of frequency up to 10^7 Hz. The temperature sensitivity of evacuated photocells is very small. The dark current (see below) is ca. 10^{-11} A [1].

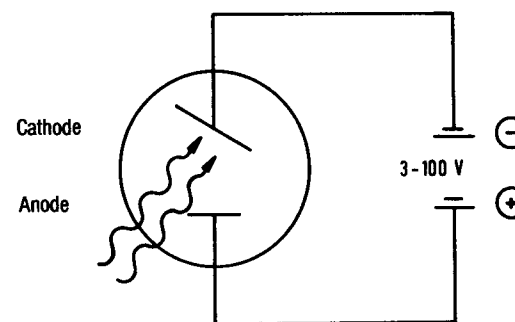


Fig. 17: The construction of a photocell, schematic [1].

Samples of analytical units with photocells:

- TURNER fluorimeter, model III (CAMAG)
- Quick Scan R & D densitometer (HELENA)
- Fiber optic densitometer, model 800 (KONTES)

Photomultipliers: Secondary electron multipliers, usually known as photomultipliers, are evacuated photocells incorporating an amplifier. The electrons emitted from the cathode are multiplied by 8 to 14 secondary electrodes (*dynodes*). A diagrammatic representation for 9 dynodes is shown in Figure 18 [5]. Each electron impact results in the production of 2 to 4 and maximally 7 secondary electrons at each dynode. This results in an amplification of the photocurrent by a factor of 10^6 to 10^8 . It is, however, still necessary to amplify the output of the photomultiplier.

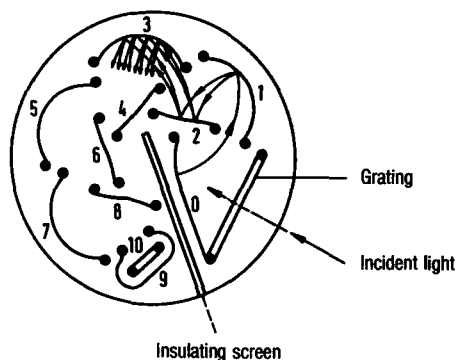


Fig. 18: Section through an RCA photomultiplier, schematic. — 0 photocathode, 1–9 dynodes, 10 anode.

The requirements for successful operation are a stable operating voltage of between 400 and 3000 V. The sensitivity of the photomultipliers is also dependent on this if a special compensation is not incorporated.

The absolute and spectral sensitivities can often vary by up to 100% within a few millimeters on the surface of the photocathode [49]. Figure 19 illustrates this effect for a sideways and vertical adjustment of a photomultiplier, in addition slight maladjustment of the light entrance can lead to “zero line runaway” as a result of thermal effects.

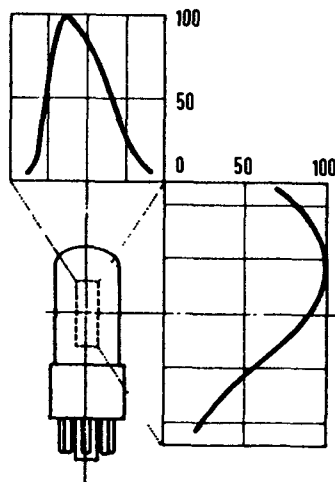


Fig. 19: Spatial dependence of the spectral sensitivity of a photocathode [50].

Depending on their positioning the dynodes are referred to as being “head-on” or “side-on”. Commercial scanners mostly employ “side-on” secondary electron multipliers where, as the name implies, the radiation impinges from the side — as in Figure 19. Their reaction time is shorter than for head-on photomultipliers because the field strength between the dynodes is greater.

Head-on photomultipliers, on the other hand, possess a greater entry angle for the capturing photocathode (Fig. 20). A diffuse screen in front of the photocathode also allows the capture of light falling at an angle. These conditions are realized in the CAMAG TLC/HPTLC scanner I. The sensitivity of such head-on photomultipliers is independent of frequency up to 10^6 Hz.

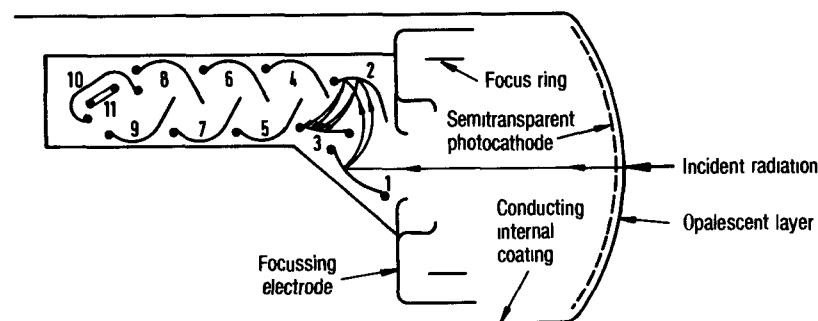


Fig. 20: Cross section through a “head on” photomultiplier [51, 52]. — 1–10 dynodes, 11 anode.

It is a disadvantage of all photomultipliers that the photocurrent is not completely proportional to the strength of illumination. Further, the photocurrent must not exceed 10^{-7} A or the photomultiplier becomes fatigued. Daylight switches are incorporated into some scanners for this reason in order to prevent over-illumination of the detector when the sample chamber is opened.

Detector noise: The detection limit for the recording of chromatographically separated substances is determined by

- the “chromatographic noise” and
- the “detector noise”.

The latter mainly results from the thermal emission current. The dark current is apparent mainly in the long-wavelength range of the spectrum when the photocurrent is appropriately small [53, 54, 131]. It is relatively small for alloy cathodes (e.g. Sb-Cs cathodes), but not small enough to be negligible.

The emission of thermal electrons is subject to statistical fluctuations (lead shot effect). It is influenced by the current strength, the number of electric charges liberated and the frequency of the radiation [55] (see [40] for further details).

Range of application and spectral sensitivity: The photomultipliers most frequently employed in scanners possess antimony-caesium cathodes. These alloy cathodes are primarily sensitive to the short-wavelength part of visible light (Tab. 4).

The *long-wavelength limit* which depends on the cathode material is ca. $\lambda = 650$ nm in the red region of the spectrum. If this does not suffice for the determination an antimony-alkaline metal alloy is employed as the cathode material [56–58].

The range of application into the *short-wavelength region of the spectrum* depends on the window material employed in the photomultiplier. Borosilicate glass (Kovar glass), for example, only transmits radiation down to about 280 nm, Suprasil down to 185 nm and fused quartz down to 160 nm. Hence fluorimeters which primarily detect long-wavelength radiation (fluorescent radiation) are often equipped with type S-4 detectors (Tab. 4), whose windows absorb a part of the short-wavelength radiations.

Table 4. Characteristics of the most important photomultipliers with Sb-Cs cathodes and 9 amplification steps [50].

Type	Origin			Spectral response [nm]	Wavelength maximum sensitivity	Window material
	RCA	HAMAMATSU	EMI			
S-4	1P21	1P21	9781A	300...650	400	borosilicate
	931B	R105	—	300...650	400	borosilicate
	—	R105UH	—	300...650	400	borosilicate
S-5	1P28	1P28	9661B	185...650	340	UV glass
	1P28/VI	R212	9781B	185...650	340	UV glass
	—	R212UH	9781B	185...650	340	UV glass
	1P28A	R454	9781R	185...650	450	UV glass
	1P28A/VI	R282	—	185...650	450	UV glass
S-19	4837	R106	9665A	160...650	340	fused silica
	—	R106UH	9783B	160...650	340	fused silica

Examples of scanners employing S-4 detectors:

- UV/VIS chromatogram analyzer for fluorescence measurements (FARRAND)
- Spectrofluorimeter SPF (AMERICAN INSTRUMENT Co.)
- TLD-100 scanner (VITATRON)

The majority of detectors, which are employed for the measurement of absorption, employ UV glass (e.g. Suprasil). All type S-5 photomultipliers possess sheaths of this material, so that they ought to be usable in the far UV region if N_2 purging is employed (to remove O_2) (Tab. 4).

Scanners with S-5 detectors

- KM 3 chromatogram spectrophotometer (C. ZEISS)
- SD 3000 spectrodensitometer (KRATOS/SCHOEFFEL)
- CD-60 densitometer (DESAGA)

Photomultipliers of type S-19 employ fused quartz instead of UV glass; this transmits down to $\lambda = 160$ nm although this far UV range is not normally employed in scanners.

Scanners with S-19 detectors

- CS 920 scanner (SHIMADZU)
- CS 930 scanner (SHIMADZU)

Photoelements and photodiodes: Both photoelements and photodiodes are photoelectric components depending on internal photoelectric effects.

In the case of *photoelements* incident quanta of light produce free charge carriers in the semiconductor layer; previously bound electrons become free. Thus, the nonconducting layer becomes conducting. In addition, the migrating electrons produce “holes” which increase the conductivity. The radiation energy is directly converted into electrical energy. The construction of a photoelement is illustrated in Figure 21.

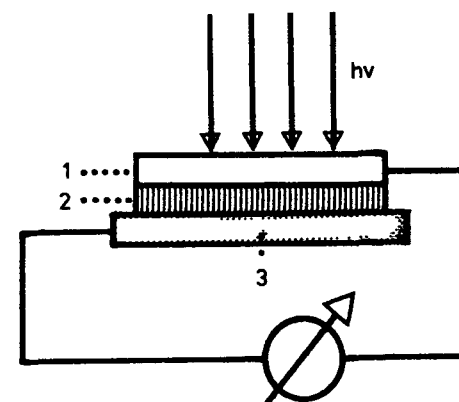


Fig. 21: Principle of construction of a photoelement [1]. — 1 light-transmitting metal layer, 2 semiconductor layer, 3 metal plate.

Photodiodes produce an electric field as a result of *pn* transitions. On illumination a photocurrent flows that is strictly proportional to the radiation intensity. Photodiodes are sensitive and free from inertia. They are, thus, suitable for rapid measurement [1, 59]; they have, therefore, been employed for the construction of diode array detectors.

2.2.3.2 Principles of Measurement

The scanners commercially available today operate on the basis of the optical train illustrated in Figure 22.

They can be employed to

- detect absorbing substances against a nonfluorescent plate background (Fig. 22A: recording a scanning curve, absorption spectra, quantitative analysis of absorbing substances);
- detect absorptions indirectly because of luminescence diminishing (Sec. 2.2.2). [Here, however, it is necessary to introduce a cut-off filter before the detector to absorb the shortwave excitation radiation (Fig. 22B)];

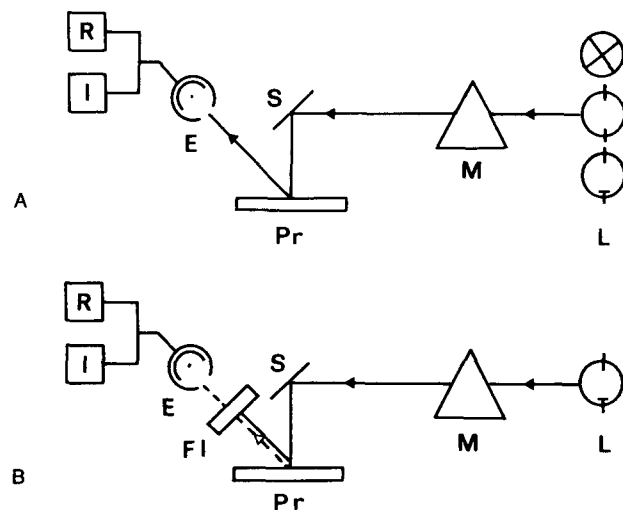


Fig. 22: Optical trains of the commercially available scanners. (A) absorption, (B) fluorescence quenching and true fluorescence.

R = recorder, I = integrator, E = detector, Pr = sample (TLC plate), S = mirror, M = monochromator, L = lamps (incandescent lamp ⊗, deuterium lamp ⊙ and mercury lamp ⊙), FI = cutoff filter.

- detect and analyze quantitatively fluorescent substances against a nonfluorescent background without spectral analysis of the fluorescent light (Fig. 22B). [An additional filter is necessary here too (Sec. 2.3.3).]

On emitting phases it is not possible to determine directly (*in situ*) the fluorescence and absorption spectra of compounds that absorb in the excitation range of luminescence indicators without distorting the measurement signal.

Direct Determination of Absorbance

Determination of absorption spectra in reflectance: If there is no luminescence radiation *absorption spectra* can be determined using the light path sketched in Figure 22A. If absorbing substances, such as, for example, dyestuffs, caffeine or PHB esters, are determined spectrophotometrically after chromatography, then, depending on the wavelength, these components absorb a proportion (I_{abs}) of the light irradiating them (I_0). The chromatographic zone emits a lower light intensity (I_{ref}) than the environment around it.

$$I_0 - I_{\text{abs}} = I_{\text{ref}}$$

Therefore it is possible to determine absorption spectra directly on the TLC plate by comparison with a substance-free portion of the layer. The wavelengths usually correspond to the spectra of the same substances in solution. However, adsorbents (silanols, amino and polyamide groups) and solvent traces (pH differences) can cause either bathochromic (ketones, aldehydes [60, 61], dyestuffs [62]) or hypsochromic (phenols, aniline derivatives [63]) shifts (Fig. 23).

However, these absorption spectra can be employed as an aid to characterization, particularly when authentic reference substances are chromatographed on a neighboring track. The use of differential spectrometry yields additional information [64]. Quantitative analysis is usually performed by scanning at the wavelength of greatest absorbance (λ_{max}). However, determinations at other wavelengths can sometimes be advantageous, e.g. when the result is a better baseline. An example is the determination of scopolamine at $\lambda = 220$ nm instead of at $\lambda_{\text{max}} = 205$ nm or of the fungicide vinclozolin at $\lambda = 245$ nm instead of at $\lambda_{\text{max}} = 220$ nm.

Absorbance (reflectance) scanning: The positions of the chromatographically separated substances are generally determined at λ_{max} . As the chromatogram is scanned the voltage differences produced at the detector are plotted as a function of position of measurement to yield an absorption scan (Fig. 24). Conclusions concerning the amount of substance chromatographed can be drawn from the areas or heights of the peaks [5].

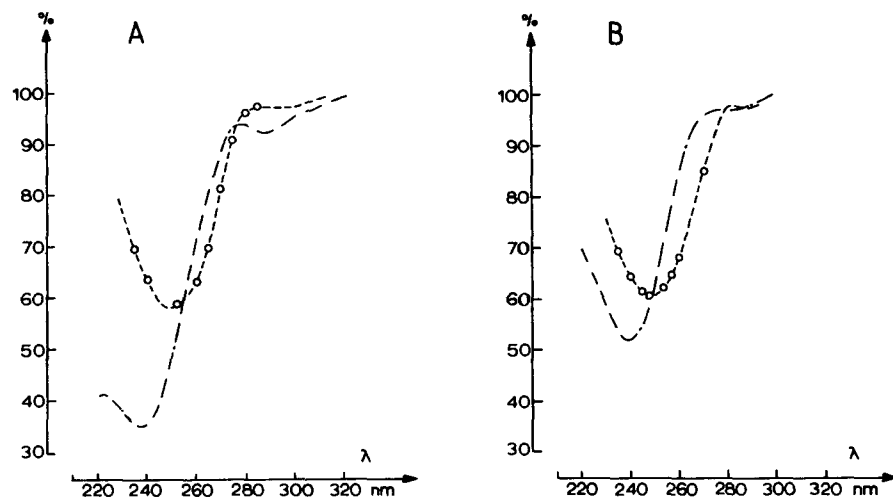


Fig. 23: Reflectance spectra (o—o—) of 3 μ g testosterone (A) and 3 μ g Δ^4 -androstendione-(3,17) (B) taken up on a silica gel layer compared with the absorbance spectra determined in methanolic solution (—)

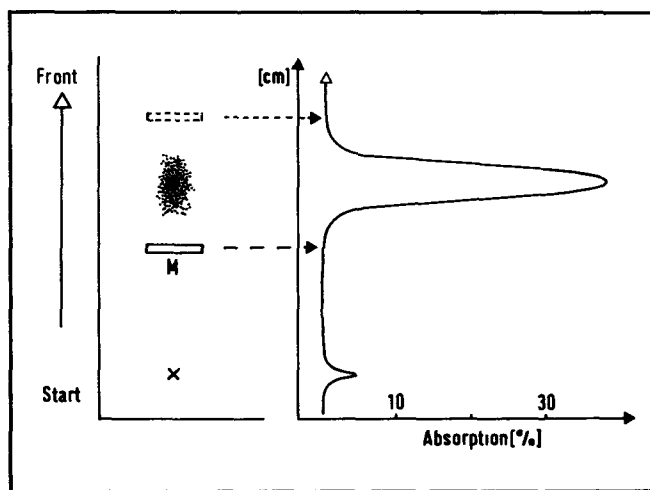


Fig. 24: Schematic representation of the recording of an absorbance scan. — M = measuring slit

Indirect Determination of Absorption (Fluorescence Quenching)

If the substance under investigation absorbs with wavelengths between 250 and 300 nm, it should be checked whether it is possible to employ chromatographic layers containing luminescence indicators. For the inorganic indicators (Sec. 2.2.2) also absorb in this range and emit, for example, yellow-green long-wavelength radiation. Hence, it is the radiation that has not been absorbed by the substance plus the fluorescence/phosphorescence radiation emitted by the indicator that arrives at the detector. The signal produced is, therefore, a composite signal of these two radiation types.

When working with a deuterium lamp the radiation energy is so low that the luminescence radiation only makes up a few percent of the total radiation. This can be easily checked in the majority of scanners by setting the total radiation (e.g. $\lambda = 260$ nm) to 100% reflectance and then inserting a cut-off filter in the beam. This filter absorbs the short-wavelength radiation before it enters the detector (Fig. 22B). The energy that remains comes from the emission of the indicator or is produced by stray light. The remaining signal is almost always small. Hence, when a deuterium lamp is employed absorption determinations are only falsified to a small extent. This falsification is also reduced by the fact that at the site of a zone the absorbing substance also reduces the emission. The absorbing substances absorb energy in the excitation range of the luminescent indicator and, hence, less light is available for the stimulation of luminescence.

However, the optical train illustrated in Figure 22B allows the determination of fluorescence quenching. The "interfering effect" described above now becomes the major effect and determines the result obtained. For this purpose the deuterium lamp is replaced by a mercury vapor lamp, whose short-wavelength emission line ($\lambda = 254$ nm) excites the luminescence indicator in the layer. Since the radiation intensity is now much greater than was the case for the deuterium lamp, the fluorescence emitted by the indicator is also much more intense and is, thus, readily measured.

The emission of the indicator is reduced in places where there are substance zones that absorb at $\lambda = 254$ nm present in the chromatogram. This produces dark zones (Fig. 4A), whose intensity (or rather lack of it) is dependent on the amount of substance applied. If the plate background is set to 100% emission the phosphorescence is reduced appropriately in the region of the substance zones. When the chromatogram is scanned peaks are produced, whose position with respect to the start can be used to calculate R_f values and whose area or height can be used to construct calibration curves as a function of the amount applied (Fig. 25).

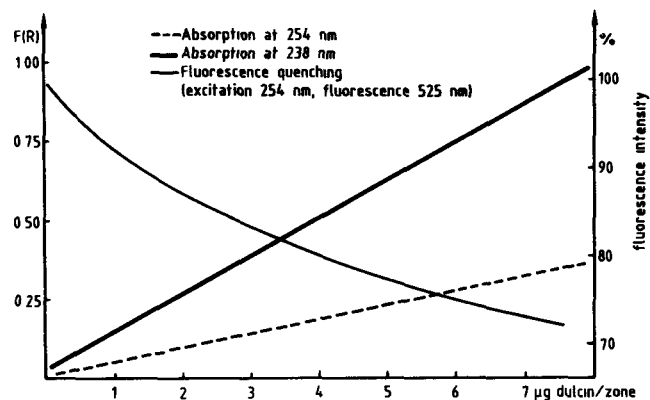


Fig. 25: Calibration curve for the determination of dulcin by fluorescence quenching and absorption [2].

However, the direct determination of absorption at the wavelength of maximum absorption is more sensitive (or in the worst case at least as sensitive) as the indirect measurement of absorption by fluorescence or phosphorescence quenching.

The fact that this type of analysis usually involves phosphorescence can be demonstrated by scanning a substance zone at various different rates. As can be seen in Figure 26 the rate of scanning of the TLC plate has an appreciable effect on the detector signal. The more rapidly the plate is moved the greater is the difference between the starting phosphorescence (chromatogram at rest) and the baseline during scanning (chromatogram in motion). Peak area and height also decrease appreciably; in addition, the baseline becomes more unsteady, which reduces the detection limit compared with that for absorption measurement at λ_{\max} . So analysis of fluorescence quenching does not provide any advantages over the direct determination of absorption.

2.2.3.3 Quantitative Analysis

The determination of the spectrum reveals which wavelength is suitable for quantitative analysis. The wavelength of maximum absorption is normally chosen, because the difference from the background blank is greatest here. In this type of analysis the analyst "sacrifices" all the substance-characterizing information in the spectrum in favor of a single wavelength. When the chromatogram is scanned photometrically at this wavelength a plot is obtained of absorption versus position on chromatogram (Fig. 25); the peak heights and areas are a function of the

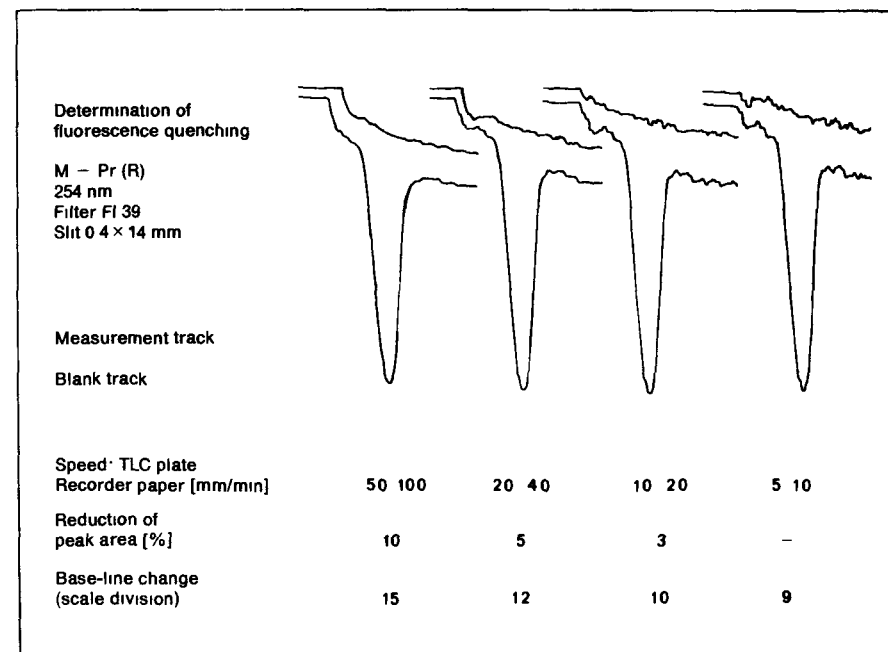


Fig. 26: Influence of scanning rate on signal size (peak area).

amount of substance applied. The limiting law is the KUBELKA-MUNK function [65, 66]:

$$\frac{k}{s} = \frac{(1 - R_{\infty})^2}{2R_{\infty}}$$

Where

- k = absorption coefficient
- s = coefficient of scattering of the stationary phase
- R_{∞} = absolute reflectance

According to the BEER-LAMBERT law

$$\frac{I}{I_0} = e^{-k \cdot d}$$

- The irradiation of the sample must be diffuse.
- Monochromatic radiation must be employed for the analysis, so that the diffraction and refraction phenomena in the layer shall be as uniform as possible. This also means that the radiation reaching the detector in reflectance retains its “color value” and only changes in its intensity. This would not be the case for polychromatic light; since a certain proportion of the light is absorbed during the determination the composition of the light would change and would, amongst other things, alter the sensitivity of the photomultiplier to the remaining light.
- Mirror reflection (= regular reflection) must not occur.
- The layer thickness must be large in comparison with the wavelength employed so that no radiation can penetrate right through the layer and escape measurement.
- The particles must be randomly distributed in the layer to avoid interference effects.
- The particles making up the adsorbent must be very much smaller in size than the thickness of the chromatographic layer.

These general requirements also apply to adsorbents laden with substance. All these requirements are not fulfilled to the same extent in thin-layer chromatography. So the KUBELKA-MUNK function does not apply without qualification.

For this reason it is understandable that numerous empirical functions have been proposed as substitutes for use in practical analysis [5, 74].

impacts into thermal energy, ... deactivation);

- emitting the absorbed energy instantan of fluorescence [3, 75, 76].

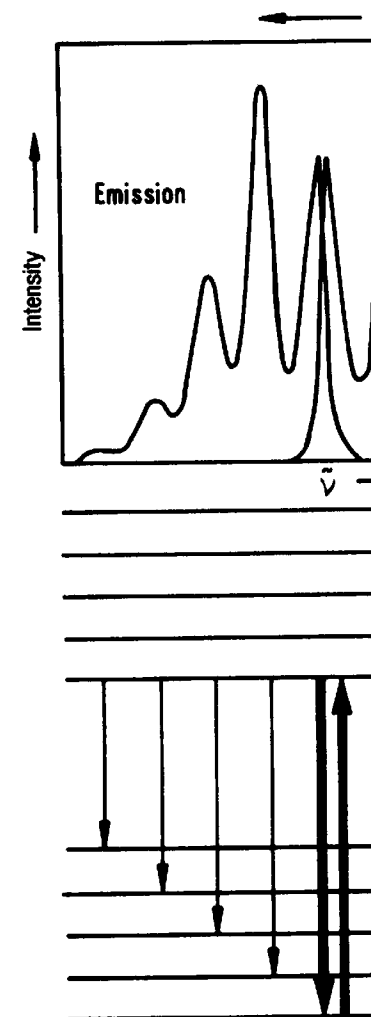


Fig. 27: Schematic representation of the relative emission of the molecules — m and m' are the numbers [4].

The radiation emitted is usually longer in wavelength (i.e. lower in energy) than the incident light (STOKE's law). It is only in the case of 0–0 transitions (shown in Figure 27 as thick arrows) that the wavelengths for fluorescence and activation are identical.

Since only relatively few substances are capable of emitting fluorescent radiation, they can be particularly selectively detected. This means that the selectivity of the chromatographic separation, which is always aimed at, is meaningfully extended by the selectivity of detection. Accompanying substances that absorb radiation but do not emit light do not interfere when the analysis is made by the selective determination of fluorescence!

The same UV lamps discussed in Section 2.2.3.1 are employed to excite fluorescence. Excitation is usually performed using long-wavelength radiation ($\lambda = 365$ nm), shorter wavelengths are occasionally employed (e.g. $\lambda = 302$ nm, DNA analysis).

2.3.2 Visual Detection

If the *fluorescent radiation* lies above $\lambda = 400$ nm it is detectable to the naked eye. Light-bright zones are seen on a dark background. When this background does not appear black this is because the "black light" filter of the UV lamp is also more or less transparent to violet visible light. This covers the whole chromatogram and also distorts the visual impression of the color of the fluorescence. Table 1 lists the emission wavelengths of the various fluorescent colors, which can, just as R_f values be employed as an aid to substance identification (in fluorimetry the subjective color impression does not correspond to the complementary color!). If the evidence so obtained is insufficient then microchemical or physiological-biological detection can follow. It is also possible to record the absorption and fluorescence spectra.

2.3.3 Fluorimetric Determinations

The optical train employed for photometric determinations of fluorescence depends on the problem involved. A spectral resolution of the emitted fluorescence is not necessary for quantitative determinations. The optical train sketched in Figure 22B can, therefore, be employed. If the fluorescence spectrum is to be determined the fluorescent light has to be analyzed into its component parts before reaching the detector (Fig. 28). A mercury or xenon lamp is used for excitation in such cases.

Cut-off filters are employed to ensure that none of the excitation radiation can reach the detector. *Monochromatic filters* are used to select particular spectral

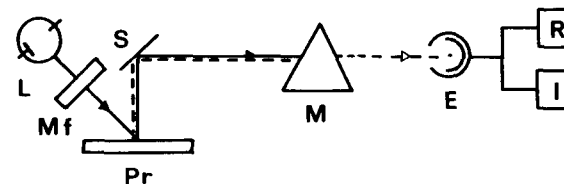


Fig. 28: Optical train for the recording of fluorescence spectra.

regions in a continuum (e.g. in the fluorescent radiation) or — employed on the excitation side — to pick out individual lines of a discontinuous spectrum. Their quality increases with the narrowness of their half-width and with their transparency at maximum.

Fluorescence scanning of chromatograms of polycyclic aromatic compounds is a vivid example of their employment. A careful choice of the wavelengths of exci-

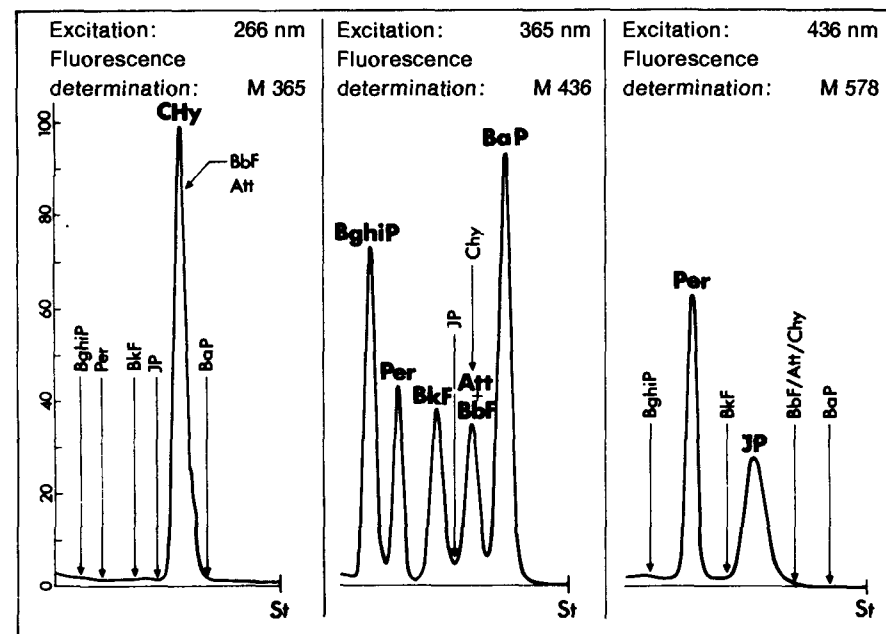


Fig. 29: Fluorescence scans of polycyclic aromatic hydrocarbons at various excitation wavelengths in combination with various secondary filters.

BghiP = benzo(g,h,i)perylene; CH_y = chrysene; Per = perylene; BbF = benzo-(b)fluoranthene; BkF = benzo-(k)fluoranthene; BaP = benzo-(a)pyrene; IP = indeno-(1,2,3-cd)pyrene; Att = anthanthrene.

tation and recording (emission) allows selective detection of the individual components (Fig 29)

The relationships between amount of substance applied and the heights or areas of the peaks in the chromatogram scan are employed for the quantitative determination of fluorescent substances. The following relationship is applicable when the amount of substance is small

$$I_{\text{fl}} = k I_0 \varepsilon a d$$

Where

I_{fl} fluorescence intensity

k proportionality factor

I_0 intensity of irradiating light

ε molar absorption coefficient

a amount of substance applied

d thickness of adsorbent layer

The intensity of the emitted fluorescence I_{fl} is, therefore, directly proportional to the amount of substance applied a . This relationship is much simpler than the KUBELKA-MUNK function and always leads to a linear calibration curve passing through the origin. If this is not true then interference is occurring [5]

When recording excitation and fluorescence spectra it must be ensured that monochromatic light falls on the detector. This can best be verified in instruments built up on the kit principle or in those equipped with two monochromators (spectrofluorimeters). The majority of scanners commercially available at the moment do not allow of such an optical train, which was realized in the KM3 chromatogram spectrometer (ZEISS). So such units are not able to generate direct absorption or fluorescence spectra for the characterization of fluorescent components.

2.4 Detection of Radioactively Labelled Substances

Radioactively labelled compounds have been employed in biology for the clarification of metabolic processes since the mid-1940s. It has, thus, been necessary to prepare such substances and to check on their purity.

All the usual detection methods are naturally suitable for the detection of such substances (Fig 2). In addition, however, it is also possible to detect the β -radiation they produce. The selectivity of the detection is, thus, increased. This, however,

does not provide any possibility of substance-specific detection, since all ^{14}C labelled compounds, for instance, give the same "reaction" at a detector.

The isotopes listed in Table 5 are the ones most frequently employed. They are all weak β -emitters with a relatively long half-life, so that they can unfortunately contaminate the organism in medical investigations.

Table 5 List of the most commonly employed β -radiation-emitting radioisotopes according to their half-lives [5]

Isotope	Energy [MeV]	Half-life
^{36}Cl	0.71	4.4×10^5 years
^{14}C	0.155	5.6×10^3 years
^3H	0.018	12.26 years
^{35}S	0.167	87.2 days
^{32}P	1.70	14.3 days
^{131}I	0.608	8.07 days

The isotopes can be detected by

- autoradiography,
- fluorography (scintillation autoradiography),
- spark chamber detection and
- scanning techniques

Their sensitivities are given in Table 6. We will discuss these detection techniques in more detail in Volume 2.

Table 6 Summary of the ^{14}C detection limits of various radio TLC methods [77]

Method	Detection limit [nCt] ^a
Liquid scintillation method	0.01
Autoradiography	0.05
Spark chamber method	0.05
Radioscanning	0.1 0.2

^a nCt symbol for nano-curie, this unit corresponds to the amount of substance which produces as many disintegrations per second as 1 ng radium.

2.5 Nondestructive Detection Using Other Physical Methods

2.5.1 Spectral Phenomena

It is often of importance to detect compounds in the chromatogram without causing chemical change. In the case of substances containing conjugated double or triple bonds observation under UV light is to be recommended (Sec. 2.2.2). If the layer contains a "fluorescence indicator" that emits radiation on irradiation with short-wavelength UV light then the zone of absorbing substance appears as a dark spot on a yellowish-green or bluish emitting background.

Substances that are intrinsically fluorescent can often be excited with long-wavelength UV light. They absorb the radiation and then emit, usually in the visible region of the spectrum, so that they appear as bright luminous zones, which can frequently be differentiated by color. They, thus, set themselves apart from the multitude of substances that only exhibit absorption. This detection possibility is characterized by high specificity (Sec. 2.3).

Differences in solubility behavior or the influence on pH are also employed for nondestructive detection and they will now be discussed.

2.5.2 Wetting and Solubility Phenomena

Silica gel, kieselguhr and aluminium oxide are hydrophilic adsorbents. Spraying or dipping the chromatogram in water yields transparent layers, on which lipophilic substances are not wetted and they appear — if their concentration is sufficient — as dry "white zones". This effect can be recognized particularly well if the TLC plate is held against the light while the completely wetted layer is slowly allowed to dry. The zones can be marked and later employed for micropreparative investigations or for biological-physiological detection. If the compounds are radioactively labelled scintillation counting can also follow [78]. Examples of such detection are listed in Table 7.

Aqueous solutions of dyes can also be employed instead of water. In the case of *hydrophilic dyes* such as methylene blue or patent fast blue the transparent background of the TLC/HPTLC plate is stained blue. Pale spots occur where there are nonwetted zones. DÄUBLE [89] detected anion-active detergents in this way on silica gel layers as pale zones on a blue background with palatine fast blue GGN

Table 7. Nondestructive detection of lipophilic substances with water as detecting reagent.

Substances	References	Substances	References
Hydrocarbons	[79]	Cholestanone, α -cholestanol	[80]
Bile acids	[81, 82]	Triterpene derivatives	[83]
Sapogenins	[84]	Cyclohexanol	[85]
N-Aryl-N',N'-dialkyl-urea		Sulfur-containing polysaccharides	[87]
herbicides	[86]		
Phosphoinositides	[88]		

and other dyestuffs. The detection limits for fatty acids lie at 1 to 2 μg per chromatogram zone [90]. The contrast is best recognizable immediately after dipping; it slowly disappears during drying.

On reversed phase layers, in contrast, detergents yield dark blue zones on a pale background. Here it is the lipophilic part of the detergent molecule that is aligned with the surface RP chain and the dye is attracted to the anionic part of the molecule. Steroid derivatives can also be detected with aqueous solutions of dyes [91].

Lipophilic dyes in aqueous alcoholic solutions can be employed in an analogous manner [92, 93]. They are enriched at the zones of lipophilic substances, so that these appear deeply colored on a pale background. This does not apply to fatty acids with less than 12 C atoms [94].

The same applies to *fluorescent substances*. These dissolve in the hydrophobic zones and lead to increased fluorescence when observed under long-wavelength UV light. MANGOLD and MALINS [95] were the first to exploit this principle. They sprayed their chromatograms with a solution of dichlorofluorescein and observed that lipophilic substances produced a yellow-green fluorescence on a purple-colored background. Their method was adopted by numerous groups of workers [96–106]. Mixtures with rhodamine B have also been employed [107, 108]. FROEHLING *et al.* [109] later employed an aqueous solution of Ultraphor WT to detect triglycerides. Glycoalkaloids can be detected fluorimetrically with alcoholic Blankophor-BA267 solution [110]. Such detection is even possible on paraffin-impregnated or RP layers [111].

Further examples illustrating the versatility of this nondestructive detection method are listed in Table 8.

Table 8. Employment of fluorescent substances for the nondestructive detection of lipophilic substances.

Reagent	Field of application and references
8-Anilidonaphthalenesulfonic acid ammonia salt (ANS reagent)	fatty acids [112, 113]; lecithin/sphingomyelin [114, 115]; cholesterol and its esters [116, 117]; steroids, detergents, hydrocarbons [118, 119]; prenol, prenylquinones [120]
Berberine	sterols [121–123]; saturated organic compounds [124]
Brilliant green	neutral esters of phosphoric acid [125], carbamate herbicides [34]
Eosin	condensation products of urea, formaldehyde and methanol [126], pesticide derivatives [127]; sweetening agents [128, 129]; anion-active and nonionogenic surface-active agents [130]
Flavonoids:	
Morin	steroids, pesticides [29, 132, 133]
Flavonol, fisetin, robinetin	pesticides [134–137]
Quercetin	vanadium in various oxidation states [138]
Rutin	uracil derivatives [139]
Fluorescein	paraffin derivatives, waxes, hydrocarbons [140, 141]; aliphatic acids [142]; hydroquinone and chlorinated derivatives [143]; isoprenoids, quinones [111, 144]; oxathizine fungicides [145]; barbiturates, phenothiazines [146]
Pinacryptol yellow	surface-active agents [147–151]; carbamate-based insecticides and herbicides [152]; organic anions [153]; sweeteners [129, 154]
Rhodamine B	vaseline [155]; diphenyl, polyphenols [156]; maleic and fumaric acids [162]; flavonoids [158]; alcohols as 3,5-dinitrobenzoates [159, 160]; gangliosides [161]; 1-hydroxychloriden [162]; carbamate pesticides [163]; parathion and its metabolites [164]; polyethylene and polypropylene glycols [165]; terpene derivatives [166]; menthol [167]
Rhodamine G	neutral steroids [168]
Rhodamine 6G	long-chain hydrocarbons [169]; squalene, α -amylin [170]; methyl esters of fatty acids [171]; glycerides [91]; sterols [172, 173]; isoprenoids, quinones [111]; lipoproteins [174]; glycosphingolipids [175]; phenolic lipids [176]; phosphonolipids [177]; increasing the sensitivity after exposure to iodine vapor [178, 179]
6- <i>p</i> -Toluidino-2-naphthalene sulfonic acid (TNS reagent)	cholesterol [180]; phospho- and glycolipids [181]; neutral lipids [182]
Uranyl acetate	purines [36]

2.5.3 Acid/Base Properties

Acidic and basic substances can be detected using pH indicators. Indicators changing color in the acid region are primarily employed. They are applied to the chromatogram by dipping or spraying with 0.01 to 1% solutions. The pH is

Table 9. Use of indicator dyes to detect pH-active substances.

Indicator/pH transition range	Application and references
Bromocresol blue (3.8...5.4)	lichen acids [186]
Bromocresol green (3.8...5.4)	aliphatic carboxylic acids [103, 187–204]; triiodobenzoic acid [205], derivatives of barbituric acid [206]; amphetamine derivatives [207, 208]; phenazones, morazone [209]; alkaloids [91, 209]; nephopam [210]; phenylamidol metabolites [211]; diethylalkylacetamide derivatives [212]; zipeprol (Mirsol) [213]; thalidomide and hydrolysis products [214]; cyclohexylamine derivatives [215]; herbicide residues [216]
Bromocresol purple (5.2...6.8)	glutamic and ketoglutaric acids [217], halide and halate anions [91, 218, 219]; preservatives [220, 221]; products of pyrolysis of epoxy resins [222]; 5-aminodibenzocycloheptane derivatives [223]; phenylalkanolamines, ephedrine [224]
Bromophenol blue (3.0...4.6)	aliphatic carboxylic acids [225–228]; malonic and lactic acids [229]; palmitic and lactic acids [230]; malonic, glycolic, malic, citric, tartaric, ketoglutaric, galacturonic and oxalic acids [196]; dicarboxylic acids, succinic acid [231]; indoleacetic acid, trichloroacetic acid [232]; palmitic acid, palmityl- and stearylactic acid [223]; benzoic, sorbic and salicylic acid [234]; metabolites of ascorbic acid [235]; chloropropionic acid [236]; oligogalacturonic acids [237]; amino acids, hydrocarbons, mono-, di- and triglycerides [238]; xylobiose, xylose, glucose and derivatives [239]; sugar alcohols [91]; toxaphene [240]
Bromothymol blue (6.0...7.6)	acid lipids, cholesterol glucuronides and gangliosides [241]; aryloxybutanolamine derivatives [242]; norfenfluramine derivatives [243]; ethylamphetamines [244]; involatile mineral oil hydrocarbons [245]; phospholipids [91]
Malachite green (0.0...2.0)	uracil derivatives, triazine herbicides [163]; polar lipids [246, 247]; phospholipids [248, 249]; fatty acids, fatty aldehydes, phospholipids and glycolipids [250]; microbiocidal isothiazolones [251]

adjusted to be near the indicator range by the addition of either boric or citric acid or borax, ammonium hydroxide or sodium hydroxide solution. Mixed indicators such as dimethyl yellow and pentamethylene red [183], or methyl red with bromocresol green [184] or with bromophenol blue [185] have also been employed in addition to the indicator dyes listed in Table 9.

2.5.4 Treatment with Iodine

Treatment of the solvent-free chromatogram with iodine vapor or by dipping in or spraying with iodine solution (0.5–1%) is a rapid and economical universal method of detecting lipophilic substances. Molecular iodine is enriched in the chromatogram zones and colors them brown.

In practice a few iodine crystals are usually placed on the bottom of a dry, closed trough chamber. After the chamber has become saturated with violet iodine vapor the solvent-free plates are placed in the chamber for 30 s to a few minutes. The iodine vapor condenses on the TLC layers and is enriched in the chromatogram zones. Iodine vapor is a universal detector, there are examples of its application for all types of substances, e.g. amino acids, indoles, alkaloids, steroids, psychoactive substances, lipids (a tabular compilation would be too voluminous to include in this section).

The chromatogram is observed and documented as soon as the spots are readily visible. The iodine can then be allowed to evaporate from the chromatogram (fume cupboard!). The chromatogram can then be subjected to further reactions or processes after this reversible reaction.

If it is desired to stabilize slightly yellow-colored iodine-containing chromatograms this is best done by treating them with dilute starch solution. This produces the well-known blue iodine inclusion compounds and these are stable over a long period.

Although, in most cases, iodine is a fairly inert halogen (in contrast to bromine) and does not normally react with the substances that have been chromatographed there are, nevertheless, examples where chemical changes have been observed. Oxidations can evidently take place (e.g. aromatic hydrocarbons and isoquinoline alkaloids [252, 253]) and additions and substitutions have also been observed. Pale zones then appear on a brown background.

Emetine and cephaeline, the two major alkaloids of ipecacuanha, begin to fluoresce after treatment with iodine vapor [254]. The molecular iodine, which acts as a quencher, must be removed by heating in the drying cupboard or on a hotplate

(fume cupboard! 60–100°C), before the yellow (emetine) and blue (cephaeline) fluorescence of the zones becomes visible. With appropriate standardization this reaction is suitable for in situ fluorimetric quantitation [255].

Other examples of irreversible derivatization on treatment with iodine have been described for phenolic steroids (estrone derivatives [256]), morphine [257] and 23 other pharmaceuticals [258]. These reactions are probably favored by the presence of silica gel as stationary phase and by the influence of light.

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3 Chemical Methods of Detection

Every analytical result forms the basis for a subsequent decision process. So the result should be subject to a high degree of precision and accuracy. This is also true of chromatographic methods. The physical detection methods described until now are frequently not sufficient on their own. If this is the case they have to be complemented by specific chemical reactions (derivatization).

These reactions can be carried out during sample preparation or directly on the layer at the start after application of the sample. Reactions have also been described in the capillaries employed for application.

Thus, MATHIS et al. [1, 2] investigated oxidation reactions with 4-nitroperbenzoic acid, sodium hypobromite, osmium tetroxide and ruthenium tetroxide. HAMANN et al. [3] employed phosphorus oxychloride in pyridine for dehydration. However, this method is accompanied by the disadvantages that the volume applied is increased because reagent has been added and that water is sometimes produced in the reaction and has to be removed before the chromatographic separation.

All cases involve prechromatographic derivatizations which introduce a chromophore leading to the formation of strongly absorbing or fluorescent derivatives

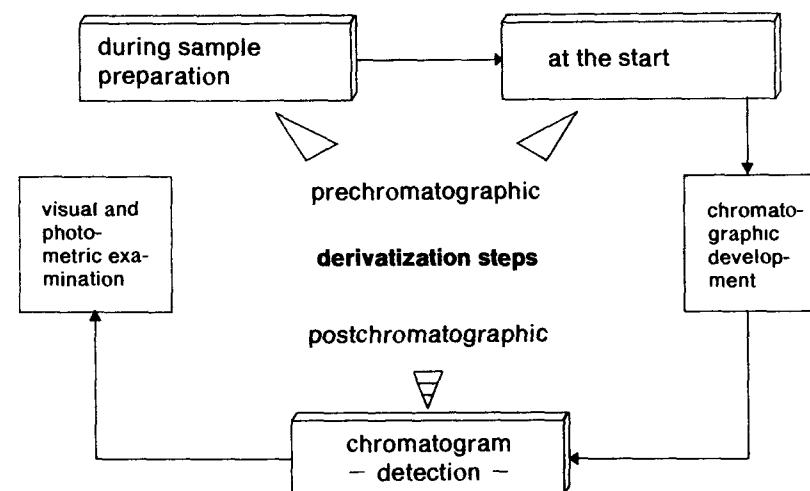


Fig. 30: Schematic representation of the position of the derivatization steps.

which increase the selectivity of the separation, increase the sensitivity of detection and improve the linearity [4]. Trace analyses often only become possible after chemical reaction of the substance to be detected. The aim of prechromatographic derivatization is, thus, rather different than that of postchromatographic derivatization, where the aim is first to detect the substance and then only secondarily to characterize it (Fig. 30).

3.1 In Situ Prechromatographic Derivatization

There has for some years been a considerable backlog in the development of practicable prechromatographic methods [5]. It is becoming more and more recognized that the future direction to be taken by trace analysts is to make improvements in the extraction, enrichment and clean-up of the sample and in the optimization of derivatization. It is only in this way that it is possible to employ the sensitive chromatographic techniques optimally for the solution of practically relevant problems.

About 100 000 new chemical compounds are synthesized every year [6]; these have to be recognized, identified and determined quantitatively. Ever more frequently this is only possible because of the employment of multiple chromatographic methods coupled with derivatization during or before the separation process.

For these reasons "Reaktions-Chromatographie" [7] ("Chromatographie fonctionnelle sur couche mince" [1, 2]) is steadily gaining in importance. Here the reaction, which also then takes on the role of a clean-up step, is performed at the start or in the concentration zone of the TLC plate.

The requirements of such a reaction are [8]:

- single, stable reaction products,
- high yields in all concentration ranges,
- simplicity and rapidity in application,
- no interference by excess reagent with the chromatographic separation and analysis that follows.

Such in situ reactions are based on the work of MILLER and KIRCHNER [9] and offer the following possibilities [10]:

- The reaction conditions can be selected so as to be able to separate substances with the same or similar chromatographic properties (critical substance pairs) by exploiting their differing chemical behavior, thus, making it easier to identify them. Specific chemical derivatization allows, for example, the esterification of

primary and secondary alcohols which can then be separated from tertiary alcohols by a subsequent chromatographic development (Sec. 3.1.5). It is just as simply possible to separate aldehydes and ketones produced by oxidation at the start from unreactive tertiary alcohols and to detect them group-specifically.

- The stability of the compound sought (e.g. oxidation-sensitive substances) can be improved.
- The reactivity of substances (e.g. towards the stationary phase) can be reduced.
- The stability of the compound sought (e.g. oxidation-sensitive substances) can be improved.

This, on the one hand, reduces the detection limit so that less sample has to be applied and, thus, the amounts of interfering substances are reduced. On the other hand, the linearity of the calibration curves can also be increased and, hence, fewer standards need to be applied and scanned in routine quantitative investigations so that more tracks are made available for sample separations. However, the introduction of a large molecular group can lead to the "equalization" of the chromatographic properties.

In practice, reaction chromatography is usually performed by first applying spots or a band of the reagent to the start zone. The sample is usually then applied while the reagent zone is still moist. Care should be taken to ensure that the sample solvent does not chromatograph the reagent outwards. The reagent solution can be applied once more, if necessary, to ensure that it is present in excess. There is no problem doing this if it is employed as a band with the Linomat IV, (Fig. 31), for instance.

If heat is necessary to accelerate the reaction, the start zone should be covered by a glass strip before being placed on a hotplate or in a drying cupboard. After reaction is complete the TLC plate should be dried and development can begin.

There have also been repeated descriptions of coupling in the sense of a two-dimensional S—R—S (separation — reaction — separation) technique. In this case the chromatogram track from the first separation serves as the start zone for a second chromatographic development after turning the plate at 90°. The derivatization described above is performed between the two chromatographic separation steps.

Reactions can also occur during chromatographic development. These can either be undesired reactions or planned derivatizations. Thus, WEICKER and BROSSMER [11] have reported, for example, that hexoses, pentoses and disaccharides can be aminated when ammonia-containing mobile phases are employed on silica gel G layers. On the other hand, fluorescamine or ninhydrin have been added to the

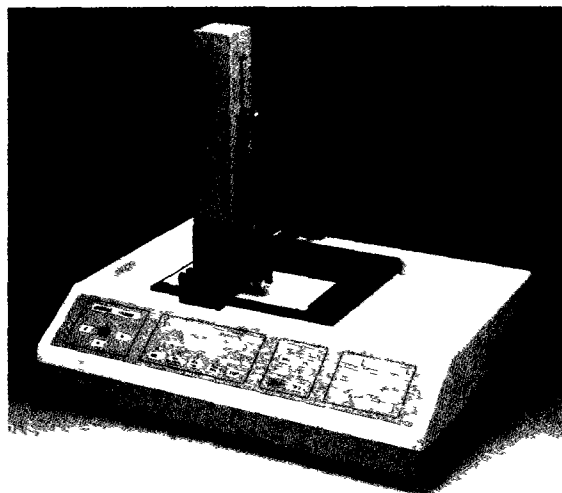


Fig. 31: Linomat IV (CAMAG).

mobile phase with the aim of converting peptides, amino acids or amines to fluorescent or colored derivatives. Unsaturated fatty acids or sterols have been brominated by adding bromine to the mobile phase or oxidized by the addition of peracetic acid. Other examples are to be found in Section 3.2.4.

When undertaking quantitative investigations it should be checked that the reaction on the TLC plate is complete — or at least stoichiometric and reproducible. In all cases it is also useful to apply reagent and sample solutions separately on neighboring tracks in order to be able to determine where the starting products appear in the chromatogram under the reaction conditions. In this way it is possible to decide whether additional by-products are produced.

3.1.1 Oxidation and Reduction

Oxidations and reductions are amongst the most frequent in situ prechromatographic reactions; they were exploited as early as 1953 by MILLER and KIRCHNER [9]. They characterized citral as an aldehyde by oxidizing it to geranic acid and reducing it to geraniol. Further examples are listed in Table 10.

3.1 In Situ Prechromatographic Derivatization

Table 10: Selection of prechromatographic derivatizations involving oxidation and reduction reactions.

Substances	Method, reagent and end products	References
Oxidations		
Phenothiazines	Apply sample solution followed by 10–20% hydrogen peroxide solution, dry at 60 °C. Sulfoxides are produced.	[12]
Anthocyanins	Anthocyanins, which interfere with the chromatographic determination of other substances, are destroyed by “overspotting” with 3% ethanolic hydrogen peroxide solution.	[13]
α -Terpineol	Apply sample solution as spots followed by 4-nitroperbenzoic acid at the start, allow to react for several minutes, dry and develop.	[1]
Geraniol	Apply alcoholic solution, then 20% chromic acid in glacial acetic acid, allow to react and develop. Citral is formed.	[14]
Isopulegol, daucol, menthol, khusol etc.	The terpenoids are applied at the start together with 1,4-naphthoquinone potassium <i>tert</i> -butoxide, heated together to 120 °C for 24 h then developed.	[15]
Diosgenin, tigogenin, androst-5-en-17-on-3- β -ol	Moisten the dried sample zone with 20% aluminium isopropoxide in benzene, spray with acetone-benzene (4 + 1), heat to 55 °C in a benzene acetone (1 + 1) atmosphere (twin-trough chamber) for 2 h, then dry, spray with 10% aqueous silver nitrate solution and finally dry for 20 min at 80 °C. Diosgenone and tigogenone are produced, for example, (OPPENAUER reaction, anhydrous solvent!).	[16]
17-Hydroxycorticosteroids	Apply sample then follow with 10% aqueous sodium periodate solution, allow to react, dry at 50 °C and develop. 17-Ketosteroids are produced.	[3]
Oleanolic acid, ursolic acid, betulic acid	Apply sample solution followed by 2% chromium(VI) oxide solution in acetic acid, spray with acetic acid and keep in an atmosphere of acetic acid for 30 to 50 min in a twin-trough chamber. Then heat to 50 °C for a few minutes, spray with methanol to destroy the excess of oxidizing agent, activate the TLC plate and develop. Oleanonic acid, 3-ketoursolic acid and 3-ketobetulic acid are produced.	[16]

Table 10 (Continued)

Substances	Method, reagent and end products	References
Alkaloids	a) 10% chromic acid in glacial acetic acid is applied on top of the sample spot. Development is performed after a brief reaction period. b) Dehydration by heating the applied sample solution on silica gel layers	[2] [18, 19]
Strychnine, brucine	Oxidation is performed with potassium dichromate solution. This oxidizes brucine to the <i>o</i> -quinone which can then be separated chromatographically	[17]
Polyaromatic hydrocarbons (PAH)	a) Apply the sample solution, spray with trifluoroacetic acid solution, heat to 100°C, cool and develop. Trifluoroacetic acid catalyzes oxidation by atmospheric oxygen. b) Apply sample solution and place in an iodine chamber for several hours, allow the iodine to evaporate. 3,4-Benzpyrene forms, for example, <i>bis</i> -3,4-benzpyrenyl	[20] [21]
Reductions		
Steroids	The applied steroids are reduced by means of a mixture of 10% ethanolic sodium borohydride solution and 0.1 N sodium hydroxide solution (1 + 1). The excess reagent is neutralized with acid after 30 min.	[3]
Strychnine	Sample applied as spots followed by 5% sodium borohydride solution, which is then dried and followed by development	[22]
Oleanolic acid, tigogenone	Apply sample and then treat with 10% sodium borohydride solution in methanol–water (1 + 5). Spray TLC plate with methanol and store in a desiccator at 55°C for 1.5 h over ethanol–methanol–dioxan (4 + 1 + 1), then dry the TLC plate (drying cupboard) and develop. Oleanolic acid and tigogenin are produced	[16]
7-Ketocholesterol, sterol hydroperoxides	The applied sample is treated with 1% methanolic sodium borohydride. After allowing reaction to proceed for 5 min the TLC plate is dried and then developed	[23, 24]
Alkaloids	Sodium borohydride solution is applied after the sample solution. The plate is dried and developed after a few minutes	[2, 25]

Table 10 (Continued)

Substances	Method, reagent and end products	References
Disulfides	The applied sample solution is treated with 0.4% sodium borohydride solution in 95% ethanol. After 15 to 20 min reaction time the excess reagent is destroyed with acid	[26]
Methyl glycyrrhetate, diosgenin	Apply 5% palladium or platinum chloride in 50% hydrochloric acid to the start, then spray with alkaline formaldehyde solution and dry in air, spray with 5% acetic acid solution and dry at 80°C. Then apply the sample solution and lightly spray with ethyl acetate. Store the TLC plate for 50 to 72 h in a desiccator over ethyl acetate in a slight stream of hydrogen, then dry and develop. The product is, for example, methyl desoxyglycyrrhetate	[16]
Fatty acids	Apply 1 drop colloidal palladium solution to the start zone and dry at 80 to 90°C for 60 min. Then apply the sample solution, store the TLC plate for 60 min in a hydrogen-filled desiccator, then dry and develop	[27, 28]
Maleic, fumaric, glutamic, citraconic, mesaconic and itaconic acid	Apply colloidal palladium solution to the starting point (diameter 8 to 10 mm) and dry. Then apply sample solution and gas with hydrogen (desiccator) for 1 h. Maleic and fumaric acids yield succinic acid etc., which may also be separated chromatographically	[29]
Amino acids	The configuration was determined by reacting with a carbobenzyloxy-L-amino acid azide and reductively removing the protective group with hydrogen/palladium chloride solution	[30]
Nitro compounds	The sample was applied, followed by 15% zinc chloride solution and dilute hydrochloric acid. The reaction was allowed to proceed for a short time, the plate was then dried and the amino compounds so formed were chromatographed	[31]
1-Nitropyrene	Extracts of diesel exhaust gases were applied to concentrating zone, platinum chloride solution was then applied followed by sodium borohydride. 1-Aminopyrene was formed	[32]
Tetrazolium salts	Formazan dyes are produced on reaction of tetrazolium salts with ammonium sulfide	[33]

Table 10 (Continued)

Substances	Method, reagent and end products	References
11- β -Hydroperoxy-lanostenyl acetate	The sample solution is applied and then treated with 5% iron(II) ammonium sulfate in water — methanol — ether (2 + 1 + 1) 11-Oxolanostenyl acetate is formed	[34]

3.1.2 Hydrolysis

Hydrolytic reactions can also be performed at the start as well as oxidative and reductive ones. They can be carried out by "wet chemistry" or enzymatically. Examples are listed in Table 11.

Table 11 Prechromatographic derivatization involving hydrolytic and enzymatic cleavage reactions

Substances	Method, reagent and end products	References
Acid hydrolysis		
Alkenylacyl- and diacylglycerol amine phosphatides	Apply bands of sample solution, overspray with 12% hydrochloric acid, leave in an atmosphere of nitrogen for 2 min and then dry in a stream of nitrogen, then chromatograph. The vinyl ether linkages in the phosphatides are hydrolyzed.	[35]
Flavone, coumarin and triterpene glycosides, solamargine, solasonine	Spray the sample zone with 10% ethanolic hydrochloric acid. Then expose to the vapors of conc hydrochloric acid — ethanol (1 + 1) in a twin-trough chamber, heat to 50 to 55°C for 4 to 5 h, dry at 90°C for 3 min, spray with 50% ethanolic ammonia solution and finally activate at 100°C for 5 min. Solasodine and the corresponding aglycones and sugars are produced.	[16]
Cardenolide glycosides	Apply bands of sample solution containing ca 25 μ g glycoside, cover the layer, apart from the application zones, with a glass plate and place in a chamber over the vapors of 37% hydrochloric acid, allow to react and chromatograph after the removal of excess hydrochloric acid.	[36, 37]

Table 11 (Continued)

Substances	Method, reagent and end products	References
Epoxides	Apply two 5 μ l portions of 10% phosphoric acid, allow to dry for 20 min then apply the sample solution and dry for 1 h. Epoxides including trisubstituted epoxides are completely ring-opened.	[38]
Sulfonamides, 1,2-di-acetylhydrazine, procaine, benzocaine, etc	Apply samples and dry, place TLC plate in a twin-trough chamber with fuming hydrochloric acid and heat to 100°C. Then remove the acid in a stream of cold air and chromatograph.	[39]

Alkaline hydrolysis

α -Amyrin benzoate, lupeol acetate, tigogenin acetate	Apply the sample solution to an aluminium oxide layer, apply 7% ethanolic potassium hydroxide solution on top and spray with methanol — water (2 + 1), then store for 4 h at 55°C over ethanol — methanol — dioxan (4 + 4 + 1), finally dry at 100°C and develop. The 3 β -alcohol is formed in each case.	[16]
<i>n</i> -Hexadecyl esters	Apply sample solution, followed by methanolic sodium hydroxide solution, warm and then chromatograph.	[40]
Phenylurea and <i>N</i> -phenyl-carbamate residues	Apply sample solution, overspot with 7% methanolic potassium hydroxide, cover the start zone with a glass plate and heat to 170°C for 20 min. Primary arylamines are produced.	[41]
Digitalis glycosides	Apply the sample solution as a band, then cover the layer, apart from the application zone, with a glass plate and place it in an ammonia chamber for 24 to 48 h, remove excess ammonia and chromatograph. Acetyl groups are split off.	[37]

Enzymatic cleavage

Cytidine-diphosphate glucose	Buffered phosphate diesterase is applied on top of the sample, covered with parafilm and warmed to 23°C for 45 to 60 min. Cytidine-5'-monophosphate and glucose-1-phosphate are formed.	[42]
Cytidine-5'-monophosphate, glucose-1-phosphate	Prostate phosphate monoesterase is employed to hydrolyze to cytidine, glucose and orthophosphate.	[42]

Table 11: (Continued)

Substances	Method, reagent and end products	References
Phosphatidylcholine	Apply phospholipase C solution as a band, dry, apply sample solution to enzyme band, stop reaction with hydrochloric acid vapor. sn-1,2-Diglycerides are produced.	[43]
Digitalis glycosides	Apply sample solution as band and then luizyme solution over it; if necessary, moisten the application zone with water. Cover the layer, except for the application zone, with a glass plate and incubate at 39°C for 2 to 5 h.	[37]

3.1.3 Halogenation

The treatment of unsaturated substances with halogen leads to addition to these molecules. This is true not only of bromine and chlorine vapor but also of the less reactive iodine. Substitution also occurs in the presence of light. Examples of such halogenations are listed in Table 12. Figure 32 illustrates the characterization of fluorescein in a bubble bath preparation. Bromination of the fluorescein in the start zone yields eosin.

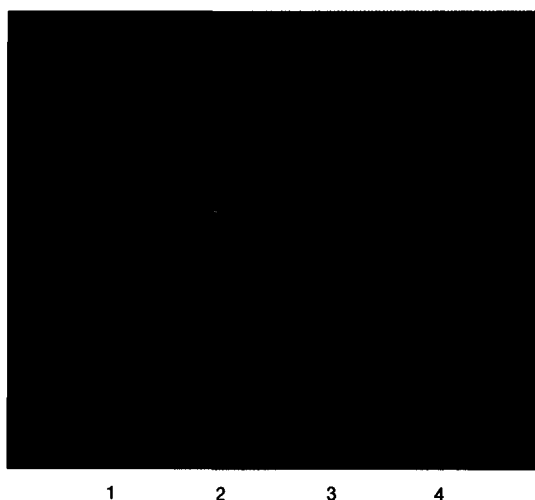


Fig. 32: Characterization of fluorescein in a foam bath by bromination. 1 = fluorescein, 2 = foam bath brominated, 3 = foam bath, 4 = eosin.

Table 12: Prechromatographic derivatization by halogenation.

Substances	Method, reagent and end products	References
Chlorination		
Cholesterol, glycyrrhetic acid acetate	Apply sample solution and moisten with anhydrous benzene, subject for 4 h to the vapors of thionyl chloride — benzene (1 + 1) in a desiccator, then dry and develop. A chlorinated cholesterol or the chloride of 3- β -acetoxyglycyrrhetic acid are formed.	[16]
Acetanilide, <i>p</i> -chloroacetanilide, 2,5-dichloroacetanilide	Apply sample solution and treat with chlorine vapor in the trough chamber for 20 s, then heat to 60°C for 5 min in a ventilated drying cupboard. Various chlorination patterns are produced.	[44]
α -Bromoisovalerylurea, acetylbromodiethylacetylurea, caffeine, codeine phosphate	Generate atmosphere of chlorine with KMnO ₄ and HCl. Expose TLC plate to chlorine for ca. 5 min. Remove excess chlorine completely by heating to 105°C for 10 min. Spray with a mixture of 100 ml 0.5% benzidine solution in ethanol and 2 ml 10% KI solution. Caffeine and codeine phosphate only react on Al ₂ O ₃ phases.	[209]
Bromination		
Cholestanol, cholesterol	Apply sample solution, treat with a 2 to 3-fold excess of 0.1% bromine in chloroform. Only cholesterol is derivatized.	[45]
Fluorescein	Apply sample solution, lead bromine vapor over it or apply 0.1% bromine in chloroform. Various intermediate bromination products are formed with eosin as the final product (Fig. 32).	[46]
Imperatorin	Apply sample solution, moisten with chloroform, place in the vapors of 10% bromine in chloroform and then dry and develop after an appropriate reaction time. Tribromoimperatorin is produced.	[16]
Sorbic acid	Treat with bromine solution or bromine vapor; di-, tri- and tetrabromocaproic acids are produced.	[47]
Capsaicinoids	Bromine vapor chamber: unsaturated capsaicinoids are completely brominated.	[48]
Phenylbutazone, prenazone	0.1% bromine in chloroform, 2 to 3-fold excess.	[49]

Table 12: (Continued)

Substances	Method, reagent and end products	References
Barbiturates, thiobarbiturates	Bromination, distinction between reacting and nonreacting barbiturates.	[50–52]
Iodination		
Pyridine, pyrrole, quino- line, isoquinoline and in- dole alkaloids	Apply sample solution and place the TLC plate in an iodine vapor chamber for 18 h, remove the excess iodine in a stream of warm air. Characterization on the basis of the iodination pattern.	[53]
Polycyclic aromatic hy- drocarbons, naphthylamines	After application of the sample solution place the TLC plate in a darkened iodine vapor chamber (azulene a few minutes, PAH several hours). Then remove the excess iodine at 60°C.	[20]
Dehydrated cholesterol	Apply sample solution; then place TLC plate in an iodine vapor chamber, blow off excess iodine. Di- and trimeric components are produced.	[54]
Phenolic steroids (estrone etc.)	Apply sample solution, then place TLC plate in an iodine vapor chamber. 2-Iodoestrone and 2,4-diiodoestrone are produced.	[55]

3.1.4 Nitration and Diazotization

Nitration and diazotization are often employed with the aim of producing colored, visually recognizable “derivatives” which are conspicuous amongst the majority of nonreacting compounds and can, thus, be specifically detected and investigated.

Aromatic nitro compounds are often strongly colored. They frequently produce characteristic, colored, quinoid derivatives on reaction with alkali or compounds with reactive methylene groups. Reduction to primary aryl amines followed by diazotization and coupling with phenols yields azo dyestuffs. Aryl amines can also react with aldehydes with formation of SCHIFF's bases to yield azomethines.

This wide range of reactions offers possibilities of carrying out substance-specific derivatizations. Some examples of applications are listed in Table 13.

Table 13: Prechromatographic derivatization by nitration and diazotization.

Substances	Method, reagent and end products	References
Nitration		
Polycyclic aromatic hy- drocarbons (PAH)	Apply sample solution and dry. Place TLC plate for 20 min in a twin-trough chamber containing phosphorus pentoxide to which 2 to 3 ml conc. nitric acid have been added. PAH nitrated by nitrous fumes.	[20]
Phenols	Apply sample solution, dry, expose to nitrous fumes.	[56]
α -, β -Naphthol, 4-chloroaniline, chlorothymol, etc.	Apply sample solution and spray with 90% nitric acid, heat to 105°C for 30 min, allow to cool and develop. Then reduce and diazotize.	[19, 57]
Marmesin, xanthotoxin	Apply sample solution and moisten with acetic acid and then expose to the vapors of conc. nitric acid and acetic acid (1 + 1) for 30 to 60 min at 55°C in a desiccator. 6-Nitromarmesin and 4-nitroxanthotoxin are formed respectively.	[16]
Brucine	Apply a drop of conc. nitric acid to the spots of applied sample solution and allow to react for 15 min. Then activate the TLC plate at 120°C for 15 min and develop after cooling.	[22]
Estrogens	Apply bands of sample solution, expose to ammonia vapor and dinitrogen tetroxide (from copper and conc. nitric acid), blow off excess and develop the nitroestrogens so formed. Detection by diazotization and coupling.	[58]
Diazotization and coupling		
<i>o</i> -, <i>m</i> -, <i>p</i> -anisidine, <i>o</i> -chloranil, 2,5-dimethoxyaniline	Apply bands of sample solution, spray with sodium nitrite in 1 mol/l hydrochloric acid solution and heat to 105°C for 5 min. After cooling apply 5% α -naphthol solution and dry in a stream of warm air. Azo dyes are formed.	[60]
2,5-Dimethoxyaniline	Apply sample solution as spots; then apply diazonium chloride and α -naphthol solution and develop after 2 min.	[61]
Estriol	Dip the concentrating zone of a precoated HPTLC silica gel 60 plate in a saturated ethanolic solution of Fast Dark Blue R salt, allow the solvent to evaporate, apply the sample solution and dip once	[10, 59]

Table 13: (Continued)

Substances	Method, reagent and end products	References
Estrone, estradiol, estrinol	again into the reagent solution; dry the chromatogram and develop it. Dip silica gel foil 2 cm in saturated Fast Black Salt K solution and dry in a stream of warm air. Apply sample solution, dip again in reagent solution and dry. Dip the TCL plate 2 cm in 4% pyridine-cyclohexane solution, dry at 100 to 200°C and develop the azo-dyestuffs that are formed.	[294]

3.1.5 Esterification and Etherification

MILLER and KIRCHNER [9] and MATHIS and OURISSON [1] have both already demonstrated that esterification at the start can be employed to distinguish primary, secondary and tertiary alcohols. Tertiary alcohols react much more slowly

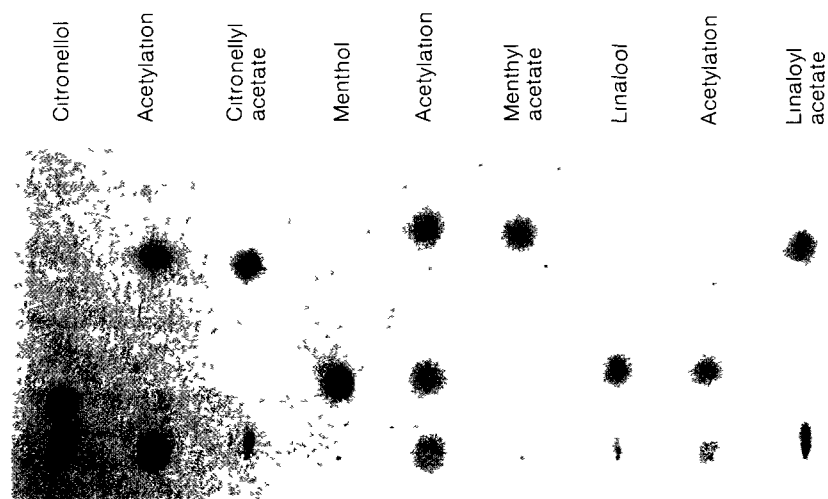


Fig 33: Differentiation of primary (citronellol), secondary (menthol) and tertiary alcohols (linalool) by in situ prechromatographic acetylation: citronellol reacts completely, menthol partially and linalool not at all.

or not at all and can, thus, be separated chromatographically from the esters that are formed (Fig. 33). Some examples are listed in Table 14. They reveal that acetic and trifluoroacetic anhydride have been employed almost exclusively for the esterification of alcohols, while acids have been esterified with diazomethane or sodium methylate.

Table 14: Prechromatographic derivatization by esterification and etherification.

Substances	Method, reagent and end products	References
Steroid sapogenins	Apply sample solution; then moisten with trifluoroacetic anhydride, dry and develop. Sapogenin trifluoroacetates are produced.	[62]
Aflatoxins	Apply extract and standard, then apply trifluoroacetic acid, allow to react at room temperature for 5 min then dry for 10 min at a max. of 40°C and develop.	[63, 64]
Aflatoxins, ochratoxin A, sterigmatocystine, penicillic acid, patulin	Apply sample solution and dry; then apply trifluoroacetic anhydride; allow to react at room temperature for 45 min, develop. The derivatives of patulin and penicillic acid possess appreciably different hR_f values.	[65]
Ochratoxin A, citrinin, penicillic acid, sterigmatocystine, zearalenone	Apply extracts of cereals or fungal cultures; apply 50 μ l pyridine — acetic anhydride (1 + 1) on top; remove the excess reagent in a stream of cold air and chromatograph. The reagents can also be applied via gas phase	[66]
Menthol, citronellol, linalool	Apply the alcohols followed by the acetylation mixture on the still damp spots. Repeated application of the reagent is necessary for complete reaction; heat to 100°C for 15 min, then chromatograph.	[46]
Polyglycerol	Apply sample solution; then apply 60% acetic anhydride in anhydrous pyridine, heat to 95 to 100°C for 15 min, allow the excess reagent to evaporate, develop.	[67]
Patulin	Apply sample solution, then apply acetic anhydride — pyridine (9 + 1), allow to react for 5 min and dry for 15 min in a stream of warm air.	[68, 69]
α -, β -Amyrin, 6-hydroxyflavones	Apply sample solution as band, followed by acetic anhydride — pyridine (6 + 1), warm to 40°C for 1.5 h in a desiccator in an atmosphere of acetic	[16]

Table 14: (Continued)

Substances	Method, reagent and end products	References
	anhydride. Then heat to 100°C for a few minutes. The corresponding acetates are produced.	
Alcohols	Apply sample solutions as bands followed by a suspension of sodium acetate in acetic anhydride — glacial acetic acid (3 + 1), then spray the plate lightly with acetic anhydride, allow to react for 3 h at 50°C in a desiccator, evaporate off and develop. Here too the acetates are produced from the alcohols employed.	[16]
Sterols, triterpenoids (e.g. lupeol), primary and secondary alcohols	Apply sample solution followed by acetyl chloride and then remove the excess reagent in a stream of hot air.	[70]
Biogenic amines (e.g. serotonin)	Apply sample solution followed by benzoyl chloride solution (5% in toluene), dry and chromatograph.	[71]
Menthol, linalool, citronellol, geraniol, α -terpineol, cinnamic alcohol etc.	Apply 10% 3,5-dinitrobenzoyl chloride solution in p-xylene — tetrahydrofuran (15 + 2), followed by sample solution; allow to react; destroy excess reagent with 10% sodium hydroxide solution.	[72]
Carboxylic acids, organophosphoric acids	Apply sample solution followed by ethereal diazomethane solution, dry and develop.	[73, 74]
Alkyl and alkenyl acylglyceryl acetates	Apply sample solution, dry, blow on hydrochloric acid vapor; methylate with 2 mol/l sodium methylate solution.	[75]
Triglycerides	Methylation with 0.5 N potassium methylate solution in methanol (transesterification).	[76]
Peanut oil, glycerol phosphatides, cholesteryl esters etc.	Apply sample solution then spray with 2 mol/l sodium methylate solution, dry for 2 to 5 min and develop. Transesterification. Only ester linkages react and not acid amide linkages.	[77]
Oleanonic acid, 6-hydroxyflavone, xanthotoxol, glycyrrhetic acid acetate	Apply sample followed by 5% potassium carbonate solution in aqueous acetone, dry, apply 50% methyl iodide in acetone. Allow to react for 3 h at 50°C in an atmosphere of methyl iodide — acetone (1 + 4), dry and develop. Oleanonic acid, for example, yields its methyl ester.	[16]
Phospholipids, free fatty acids	Apply sample solution, then 12% methanolic potassium hydroxide solution, keep moist with methanol for 5 min. Fatty acid methyl esters are produced, triglycerides do not react.	[7]

Table 14: (Continued)

Substances	Method, reagent and end products	References
Fatty acids, <i>n</i> -hydroxy acids, ursolic acid	Apply sample solution, followed by methanolic boron trifluoride solution, heat with a hot-air drier, allow to cool and develop.	[70]
Sorbic acid, benzoic acid	Apply sample solution in the form of a band, followed by 0.5% 4-bromophenacyl bromide in <i>N,N</i> -dimethylformamide. Heat to 80°C for 45 min, dry and chromatograph.	[78]
Phenols	Apply sample solution and spray with saturated sodium methylate solution and then treat with 4% 2,4-dinitrofluorobenzene in acetone and heat to 190°C for 40 min. Chromatograph the dinitrophenyl ethers so produced.	[79]

3.1.6 Hydrazone Formation

In order to characterize them and more readily separate them from interfering accompanying substances carbonyl compounds (aldehydes, ketones) can be converted to hydrazones at the start. The reagent mainly employed is 2,4-dinitrophenylhydrazine in acidic solution [70]. This yields osazones with aldoses and ketoses. Some examples are listed in Table 15.

Table 15: Prechromatographic derivatization by hydrazone formation.

Substances	Method, reagent and end products	References
Dipterocarpol, hecogenin, progesterone	Apply sample solution and moisten with 2,4-dinitrophenylhydrazine in acetic acid; then spray with acetic acid and store at room temperature or 55°C for up to 1.5 h in a desiccator; then dry at 80°C and chromatograph.	[16]
Progesterone	Apply sample solution as a band followed by 0.1% ethanolic 2,4-dinitrophenylhydrazine (acidified with 0.1% conc. hydrochloric acid), allow to react at room temperature for 10 min and dry at 100°C for 5 min.	[80]

Table 15 (Continued)

Substances	Method, reagent and end products	References
Steroid ketones	Apply the sample solution followed by GIRARD's reagent (0.1% trimethylacetyl hydrazide in 10% acetic acid) and allow to react for 15 h in an atmosphere of acetic acid. Then dry at 80°C for 10 min and after cooling chromatograph the hydrazones that have been formed.	[81]
Aldehydes, ketones	Apply sample solution and moisten with 2 N 2,4-dinitrophenylhydrazine in acetic acid. After reacting, dry and chromatograph the 2,4-DNPH derivatives.	[14]
Carvone, menthone, acetophenone etc	Apply an acidic solution of 2,4-dinitrophenylhydrazine, 4-nitrophenylhydrazine or 2,4-dinitrophenylsemicarbazide onto the previously applied sample solution. Aliphatic and aromatic hydrazones and carbazones can be differentiated by their colors.	[82]
Phenolic aldehydes	Apply sample solution and then acidic 2,4-dinitrophenylhydrazine solution, allow to react, dry and develop.	[15]
Chloro-, hydroxy-, and methoxybenzaldehyde derivatives	Derivatize with 2,4-dinitrophenylhydrazine solution in hydrochloric acid. Heat to 80°C, cool and chromatograph.	[83]
<i>p</i> -Benzoquinone derivatives	Derivatization with 2,4-dinitrophenylhydrazine solution in hydrochloric acid. Heat to 80°C, cool and chromatograph.	[84]
<i>o</i> -, <i>m</i> -, <i>p</i> -chloro-, 2,4- and 3,4-dichlorobenzoic acids	Apply <i>p</i> -bromophenacyl esters of the substances, followed by 0.5% 2,4-dinitrophenylhydrazine in 2 mol/l hydrochloric acid and allow to react for 10 to 15 min in the desiccator.	[85]

3.1.7 Dansylation

Prechromatographic dansylation has the advantage that chromatography separates excess reagent and also the fluorescent by-products (e.g. dansyl hydroxide) from the reaction products of the substances to be determined. In the case of postchromatographic dansylation the whole of the plate background fluoresces blue, so that in situ analysis is made more difficult.

Primary and secondary amines, amino acids and phenols react. In the case of long-wavelength UV light ($\lambda = 365$ nm) the DANS amides fluoresce yellow-green, while amines that have reacted at a phenolic OH group have an intense yellow to yellow-orange fluorescence. The detection limit for DANS amides is ca. 10^{-10} mol [86].

Acids can also be converted to fluorescent dansyl derivatives. The reaction of C_8 to C_{24} fatty acids with dansyl semipiperazide or semicadaveride provides an excellent example (Fig. 34) [87]. Odd-numbered and unsaturated fatty acids [88] and propionic, sorbic and benzoic acid [89] can be detected in the same manner.

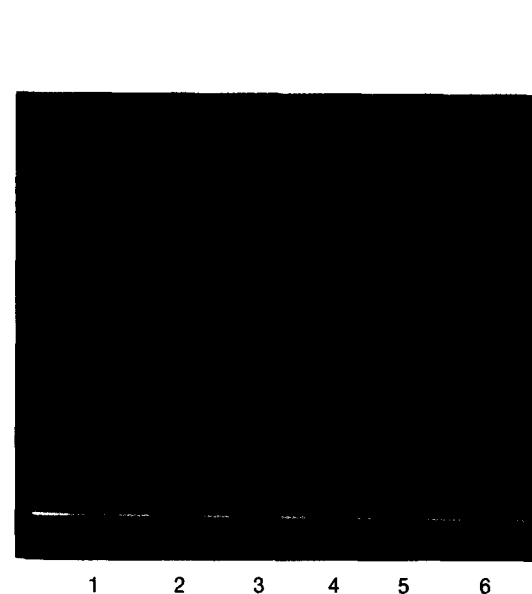


Fig. 34

Fig. 34: Chromatogram of various even- and odd-numbered fatty acids after in situ derivatization with dansyl semicadaveride. The separations are with increasing R_f — track 1: C-24 to C-16, track 2: C-24 to C-6, track 3: C-20 to C-12, track 4: C-20 to C-11, track 5: C-19 to C-11, track 6: C-24 to C-16.

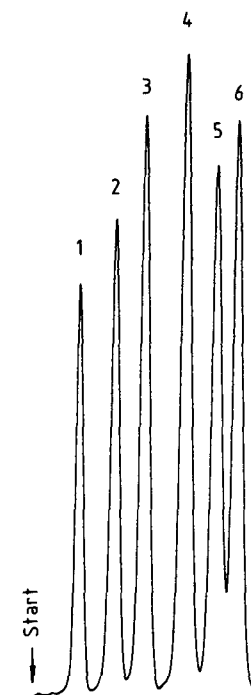


Fig. 35

Fig. 35: Fluorescence scan of the dansyl semicadaveride derivatives of 1: behenic acid, 2: erucic acid, 3: stearic acid, 4: oleic acid, 5: linoleic acid, 6: linolenic acid.

Table 16 lists some examples of dansylation

Table 16 Prechromatographic derivatization by dansylation

Substances	Method, reagent and end products	References
Carbamate and phenylurea herbicides	Treat the sample for 30 to 40 min with sodium hydroxide solution ($c = 1$ mol/l) at 80°C. Apply sample solution and then 0.2% dansyl chloride over it. Cover with a glass plate and allow to react in the dark at room temperature for 60 min, then chromatograph	[90]
Phenylurea herbicides	Treat sample with KOH, apply sample solution and overspot with dansyl chloride. Allow to react for 30 min in the dark at room temperature, then develop the chromatogram	[91]
Metoxurone and degradation products	Phenylurea herbicides are first hydrolyzed to the corresponding aniline derivatives and then reacted at the start with 4 μ l 0.25% dansyl chloride solution	[92]
Urea herbicides, e.g. diuron, metoxurone, linuron	Apply sample solution to silica gel layer, cover with a glass plate, heat to 160°C for 25 min and allow to cool. After this hydrolysis apply 0.2% dansyl chloride in acetone, cover with a glass plate and store in the dark for 1 h, then chromatograph	[93]
Morphine, 6-mono-acetylmorphine, morphine-6-nicotinate	Apply sample solution and apply to each spot 1 μ l of dansyl chloride and two times 1 μ l 8% sodium bicarbonate solution, allow to react for 7 min, dry at 70°C and develop the chromatogram	[94–96, 237]
β Blockers	Apply the urine extract in the form of a band, overlay with 0.1% dansyl chloride in acetone followed by 8% aqueous sodium bicarbonate solution and heat to 120°C for 15 min, allow to cool and develop the chromatogram	[97]
Serum proteins, e.g. albumin	Allow to react at the start with 0.05% dansyl chloride solution in hexane, dry and expose for 10 h to the vapors of a triethylamine bicarbonate buffer ($c = 0.1$ mol/l, pH 8.5), then develop. Excess reagent and by-products run with the solvent front	[98]
Even-numbered and odd-numbered fatty acids (C_6 – C_{24})	Apply dansyl semipiperazide or dansyl semicadaveride solution as a 14 cm long band, followed by sample solution as short bands and then	[87, 88]

Table 16 (Continued)

Substances	Method, reagent and end products	References
	1% <i>N,N'</i> -dicyclohexylcarbodiimide solution as a 14 cm band. Dry and develop (Figs 34, 35 and title picture)	
Preservatives (benzoic, sorbic, propionic acid)	Apply sample solution as spots or bands (3 to 4 mm) followed by dansyl semipiperazide and <i>N,N'</i> -dicyclohexylcarbodiimide solution, dry well and chromatograph	[89]

3.1.8 Miscellaneous Prechromatographic Derivatizations

Other group-characteristic *in situ* prechromatographic reactions have been described in addition to the ones discussed above. They all serve to improve the characterization of the substances concerned and the selectivity of the subsequent chromatography. Table 17 provides an overview.

Table 17 Miscellaneous prechromatographic derivatizations

Substances	Method, reagent and end products	References
Amino acids	Apply sample solution, dry, treat with 2,4-dinitrofluorobenzene solution. DNP-amino acids are produced, which are then separated chromatographically	[99]
2 Anilino-5-thiazolinone derivatives of amino acids	Apply sample solution followed by heptafluorobutyric acid, heat to 140°C for 10 min, rapidly cool to room temperature and develop the phenylthiohydantoin that are formed. No reaction occurs with, for example, threonine, serine, tryptophan or glutamic acid	[100]
Amines	Apply sample solution and spray with 10% carbon disulfide in ethyl acetate. Place TLC plate in carbon disulfide vapor for 30 min, then spray with methanol – sulfuric acid (1 + 1). Heat to 100°C for 10 min and chromatograph the isothiocyanates so formed	[101]

Table 17 (Continued)

Substances	Method, reagent and end products	References
Amines	Apply sample solution then <i>p</i> -toluenesulfonic acid in pyridine, heat to 60°C for 4 h, after cooling to room temperature develop the <i>p</i> -toluenesulfonates so formed	[102]
Serotonin	Benzoylate the amino groups by overspotting at the start This makes detection with GIBBS' reagent possible	[103]
Sympathomimetics with free amino groups e.g. carbadrine, norfenefrine, noradrenaline, norephedrine	Apply sample solution (50 pg to 300 ng) Then apply fluorescamine (0.03% in acetone), dry and chromatograph	[104]
Amines, Amino acids, peptides, e.g. tryptophan, tryptamine, peptides with terminal tryptophan groups	Apply indole derivatives dissolved in sodium borate buffer solution ($c = 0.2$ mol/l, pH 9.0) – ethanol (1 + 1) Dip TLC plate in fluorescamine solution to just above starting zone (15 s) Then dry at room temperature and develop In case of indole amines followed by spraying with 40% perchloric acid	[105]
Catecholamines	Apply sample solution followed by phosphate buffer ($c = 0.5$ mol/l, pH 8.0) Dip TLC plate into fluorescamine solution to just above starting zone, dry and develop Blue fluorescence occurs after spraying with perchloric acid (70%)	[105]
Habituating drugs	Apply sample solution Let it react with NBD-chloride or diphenylacetyl-1,3-indandion-1-hydrazone	[236]
Desoxyribo-oligonucleotides, ribopolynucleotides	Layer PEI cellulose Complex formation of polyuridylic acid (6 mg/ml) with desoxyadenosine oligonucleotides	[106]
Aliphatic and aromatic aldehydes	Apply sample solution, followed by 1% aniline in dichloromethane and chromatograph the SCHIFF'S bases after 10 min	[107]
Insecticides, e.g. eldrin, dieldrin, aldrin	Apply the sample solution, dry and then apply ethanolic zinc chloride solution, heat to 100°C for 10 min, after cooling chromatograph the carbonyl compounds that have formed	[108, 109]
Carveol, linalool, geraniol, α -terpineol, nerol etc	Elimination of water with the aid of sulfuric acid and formation of the corresponding monoterpene hydrocarbons	[9, 14]

Table 17 (Continued)

Substances	Method, reagent and end products	References
Alcohols	Apply sample solution, then nitrophenyl isocyanate solution (10% in benzene) Dry after reacting and develop	[72]

Other possibilities are the reduction of nitro groups by applying the sample solutions to adsorbent layers containing zinc dust and then exposing to hydrochloric acid vapors [110]. 3,5-Dinitrobenzoates and 2,4-dinitrophenylhydrazones can also be reduced in the same way on tin-containing silica gel phases [111]. Cellulose layers are also suitable for such reactions [112]. SEILER and ROTHWEILER have described a method of “trans-salting” the alkali metal sulfates to alkali metal acetates [113].

3.2 Postchromatographic Detection

There is no difficulty in detecting colored substances or compounds with intrinsic fluorescence on TLC chromatograms. The same applies to components absorbing in UV light which have been separated on layers with incorporated “fluorescence indicators” and, hence, cause phosphorescence quenching in UV light so that the substances appear as dark zones on a bright emitting background.

Substances which do not exhibit such properties have to be transformed into detectable substances (derivatives) in order to evaluate the TLC separation. Such reactions can be performed as universal reactions or selectively on the basis of suitable functional groups. Substance-specific derivatization is practically impossible.

The aim of a postchromatographic derivatization is first

- the detection of the chromatographically separated substances in order to be able to evaluate the chromatogram better, visually

But equally important are also

- increasing the selectivity, which is often associated with this and

- improving the detection sensitivity In addition comes optimization of the subsequent in situ quantitation

The separation is already complete when detection is undertaken. The solvent has been evaporated off, the substance is present finely distributed in the adsorbent. For a given amount of substance the smaller the chromatogram zone the greater is the concentration and, hence, the detection sensitivity. For this reason substances with low R_f values are more intensely colored than those present in the same quantity which migrate further.

Each reaction requires a minimum concentration for the detection to be possible at all. This concentration naturally varies from reagent to reagent (Fig 36), so that every component cannot be detected to the same sensitivity with every reagent. This can lead to the appearance of a good separation that is, in fact, not good at all because the outer edge of a zone is not detected. So that the area occupied by the substance appears to be smaller than it actually is. The separation of the neighboring zones will, thus, seem better. It follows that the least sensitive reagents can counterfeit the "best separations"

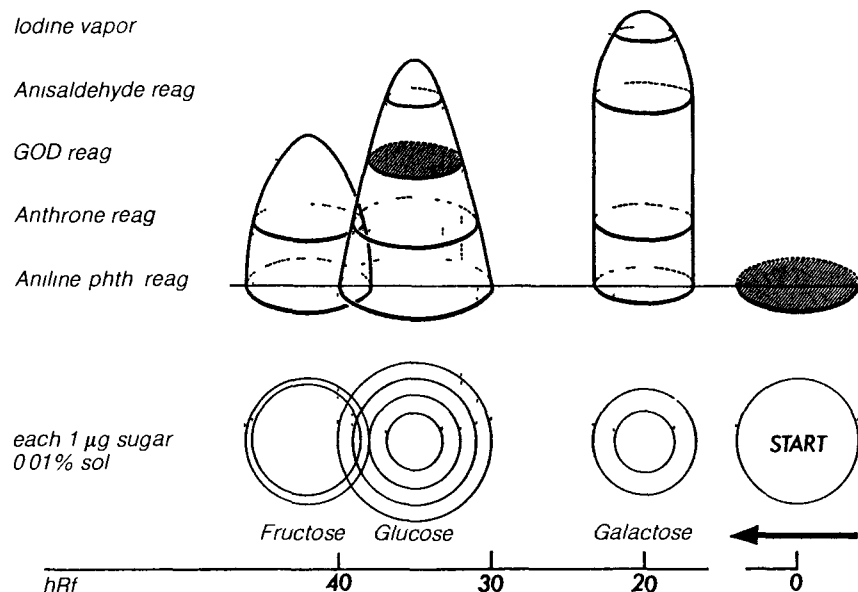


Fig. 36: Dependence of the area of the chromatogram zone at constant amount of applied substance (1 μ g) on the reagent employed, top: relief representation, below: zone areas projected on one another. Iodine vapor reacts least sensitively here, aniline-phthalate most sensitively and the GOD reaction (glucose oxidase reaction) most specifically [216]

More sensitive detection methods and more objective recording methods (e.g. the employment of scanners) are constantly being striven for in order to overcome this illusion. It is for this reason too that fluorescent methods have been introduced to an increasing extent on account of their higher detection sensitivity. This allows an appreciable reduction in the amount of sample applied, so that possible interfering substances are also present in smaller quantities. This increases the quality of the chromatographic separation and the subsequent in situ analysis.

Various techniques are employed for applying the reagents to the TLC/HPTLC plate. The least satisfactory is spraying the reagent manually onto the chromatogram (Fig 37). Dipping and evaporation methods are preferable with respect to precision and repeatability (Fig 38). Methods have also been developed and described involving the addition of the reactants to the mobile or the stationary phase. These application techniques will be described below before discussing the influence of temperature on the reaction.

3.2.1 Spraying

Until a few years ago the most common method of rendering colorless substances on chromatograms visible was to spray them with reagent solutions [115]. An all-



Fig. 37: Manual spraying of the chromatogram

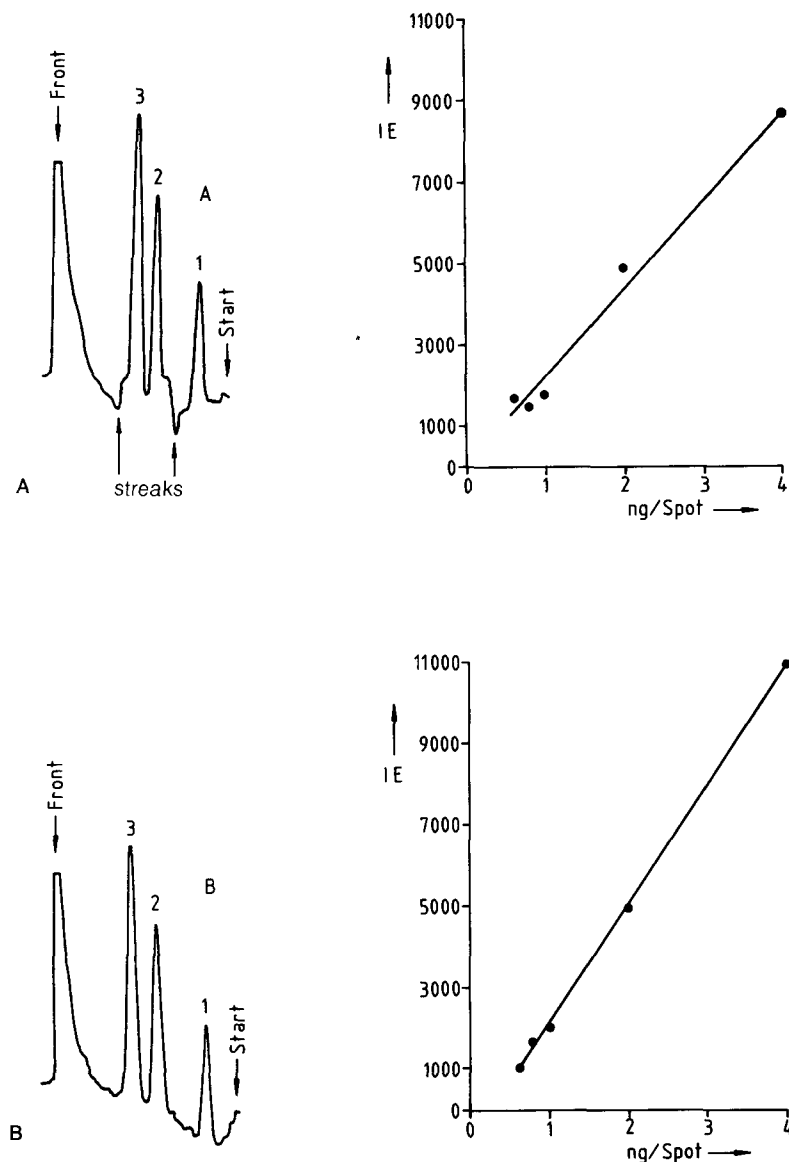


Fig. 38: Comparison of manual dipping (A) with mechanized dipping (B) on the basis of scans and calibration curves [114] — 1 = *cis*-diethylstilbestrol, 2 = *trans*-diethylstilbestrol, 3 = ethinylestradiol. Scanning curve 2 ng of each substance per chromatogram zone $\lambda_{exc} = 313 \text{ nm}$, $\lambda_{fl} > 390 \text{ nm}$. Dipping solution: water — sulfuric acid — methanol (85 + 15 + 1)

glass sprayer was normally employed (Fig. 39A), which was connected to a pressure supply (membrane pump) or cylinder of inert gas. It was necessary to use a jet so fine adjusted that it was possible to spray the reagent solution homogeneously. Spraying was carried out at a pressure of 0.6 to 0.8 bar from a distance of 20 to 30 cm in a suitable fume cupboard (Fig. 37). According to WALDI the spray should be applied in a meandering pattern with the return point of the spray outside the track of the chromatogram (Fig. 39B) [115].

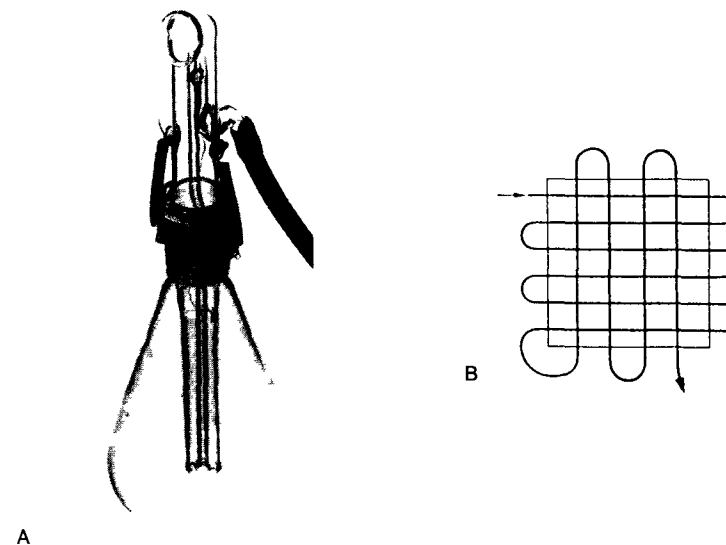


Fig. 39: All glass sprayer (A), spray pattern (B)

The same applies to the use of spray pistols (spray guns and aerosol cans), the frequency of whose use ought probably to be reduced on account of the propellant gas (chlorofluorohydrocarbons) employed. Manual depression of the button valve of the vertically held spray can "shoots" the propellant gas through a fine jet and drags the sucked-up reagent solution with it onto the vertically held chromatogram (water pump principle).

Because when operated manually the spray unit can never be moved so uniformly that the chromatogram is homogeneously covered with reagent and the amount

of reagent applied to the TLC plate differs for each individual, SPITZ [116], KREUZIG [117–119] and others have developed and marketed automatic sprayers. In the first case the sprayer is moved over the fixed, stationary TLC plate. The second type of automatic sprayer works with a fixed spray jet and the TLC plate is moved by a motor.

Neither apparatus has, as yet, found general use in the laboratory, probably because too little attention was paid to the differing viscosities, surface tensions and polarities of the various solvents. Thus, in practice, these automatic sprayers repeatedly produced “sprinkled” zones because, in addition, to a fine mist individual larger drops also reached the zones and, thus, caused inhomogeneities of coverage and reaction and also because substances at the surface of the layer, where they are to be found after chromatography with readily volatile mobile phases [120–124, 128], are displaced in the direction of the supporting backing, so that in extreme cases detection from the rear is more sensitive than from the front.

The spraying of TLC/HPTLC plates should always be undertaken in a well-ventilated fume cupboard, so that the aerosols, some of which are damaging to health and aggressive, are not breathed in and the place of work is not contaminated. After the spraying is complete the spraying apparatus and fume cupboard should be cleaned with care so that undesired reactions do not occur with later reagents. Manganese heptoxide and perchloric acid-containing reagents, sodium azide and iodine azide solutions should never be sprayed as they can cause explosions in the exhaust ducts of fume cupboards. For the same reasons such reagents should only be made up in small quantities. In all these cases it is preferable to apply the reagent by dipping the chromatogram into it.

3.2.2 Dipping

It is becoming ever more usual to dip the solvent-free chromatograms into a suitable reagent solution [114]. The reasons for this are:

- The coating of the adsorbent layer with the reagent solution is more homogeneous than with even the most carefully carried out spraying process.
- The distribution of the reagent is no longer influenced by the manual dexterity of the operator, the performance of the spray apparatus, the viscosity of the reagent or the drop size of the spray mist.
- It is only since this method has come into use that the precise quantitative analysis of thin-layer chromatograms has become possible. For the increased

regularity of the wetting results in a baseline with less structure (Fig. 38B). This means that the detection limits are appreciably lower than they are in the case of sprayed chromatograms. The reproducibility of the result is also appreciably improved on account of the homogeneity of the reagent application (Tab. 18).

- The consumption of reagents is less particularly when series investigations are made (when the reagent is used repeatedly it is usual to cover the dipping chamber with a stainless steel lid).
- The contamination of the place of work with reagents that may be injurious to health or corrosive, is considerably less when dipping than when spraying.
- The complex spray facilities with integrated fume cupboard are unnecessary.

In spite of these acknowledged advantages, low volume dipping chambers and automated, time-controlled dipping apparatus (Fig. 40 and 41) have only been available commercially for a few years.

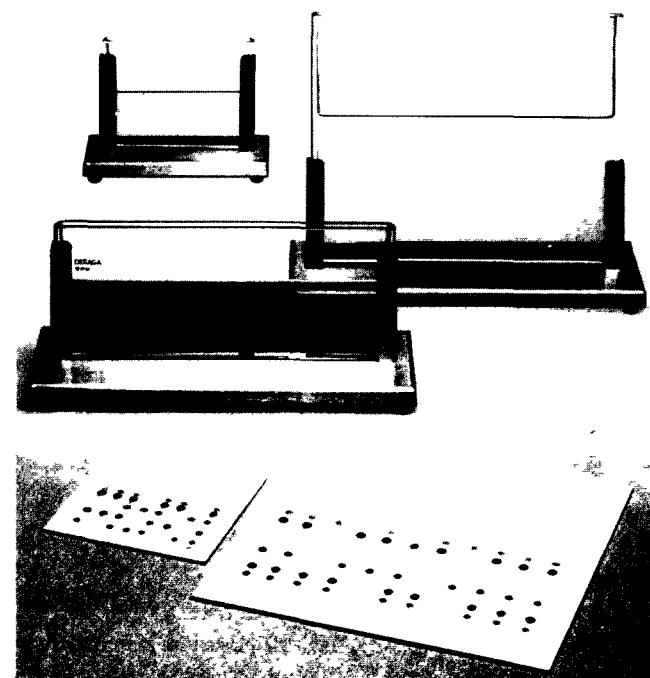


Fig. 40: Low volume dipping chambers (DESAGA).



Fig. 41: Automated dipping devices (DC Tauch-fix, BARON).

The advantage of such dipping apparatus is that the insertion and removal of the chromatogram is performed at a uniform speed and the time of immersion can be set as necessary. Interfering “ripple marks”, such as are observed on manual dipping, do not occur. Care must be taken, however, to clean off the back of the

Table 18. Statistical comparison (F-test [125]) of the methods. Standard deviation s_{x0} of the calibration curves for diethylstilbestrol and ethinylestradiol [114].

Procedure	<i>trans</i> -diethylstilbestrol s_{x0} [ng per zone]	Ethinylestradiol s_{x0} [ng per zone]
Manual dipping	0.37	0.21
Mechanical dipping	0.096	0.044
Statistical difference (F-test)	significant	significant

TLC plate which is wetted with reagent solution when it leaves the dipping bath before laying it on the hotplate, laboratory bench or scanning stage.

The dipping solutions described in Part II of this book are usually less concentrated than the corresponding spray solutions. The solvents employed are specially chosen for their suitability to the special requirements of dipping solutions. Water, which on the one hand, can sit on the surface of RP plates and not penetrate them and, on the other hand, can cause disintegration of water-incompatible layers is usually replaced by alcohol or other lipophilic solvents.

In general care should be taken in the choice of solvent to ensure that neither the chromatographically separated substances nor their reaction products are soluble in the solvent of the dipping reagent.

It is probable that the solvents given in the individual reagent monographs are not suitable for all the substances with which the reagent will react. This point should be taken into account especially for quantitative work and the user should make appropriate modifications. In particular, there must be no loss of substance or reaction product by dissolution (formation of “comet tails” by the chromatographic zones).

When the plate is inspected the color intensity of the chromatogram zones must be more intense at the top surface of the layer than it is when viewed from the back of the TLC/HPTLC plate. If this is not the case the reagent must be made less polar to avoid a frontal development across the thickness of the layer.



Fig. 42: Chromatogram of polycyclic aromatic hydrocarbons on caffeine-impregnated pre-coated silica gel 60 HPTLC plates with concentrating zone (MERCK). The following can be recognized in increasing R_f value. — 1. benzo(ghi)perylene, 2. indeno(1,2,3-cd)pyrene, 3. benzo(a)pyrene, 4. benzo(b)fluoranthene, 5. benzo(k)fluoranthene, 6. fluoranthene.

The times of immersion of the chromatogram in the reagent bath are usually short (< 5 s [126]) in order to avoid dissolving the substances out of the stationary phase. This is easily achieved if the Tauch-Fix (Fig. 41) is employed. The chromatogram is then laid horizontally and dried in a stream of air.

The dipping unit can also be employed to impregnate adsorbent layers. It is easy in this way to produce tungstate- [127] or silver nitrate-impregnated layers for separating oligosaccharides or unsaturated compounds.

FUNK et al. [128a] dipped silica gel plates in a 4% solution of caffeine in order to separate six polyaromatic hydrocarbons relevant in monitoring the quality of potable water (Fig. 42).

Such a dipping apparatus can also be employed with advantage for applying substances to preserve or intensify fluorescence after chromatography or derivatization is complete (cf. Section 3.2.7.3).

3.2.3 Exposure to Vapors

A layer can also be homogeneously treated with the reagent by exposure to its vapor. This is frequently carried out in a twin-trough chamber (Fig. 43A) or in a special conditioning chamber (Fig. 43B). In the case of the twin-trough chamber, for example, the reagent is placed in one of the troughs and the dried chromatogram plate in the other. Thin-layer chromatograms can be treated in this manner with the vapors of a large number of reagents.

Iodine vapor allows nonspecific, usually nondestructive detection of many substances (e.g. surface active agents [129], pharmaceuticals [130, 131], polyethylene glycols [132], see also Table 12). In addition, reactions have also been described with the vapors of bromine [133–135], cyanogen bromide [136], chlorine [137–141, 209], ammonia [142–147] (see also the reagent “Ammonia vapor”), diethylamine [148], ammonium hydrogen carbonate [149, 150], acids [145, 151–156] (see also reagent “Hydrochloric Acid Vapor”), *tert*-butyl hypochlorite [203], sulfuryl chloride [157, 158], sulfur dioxide [159, 160] oxides of nitrogen [161–169], hydrogen sulfide [170], formaldehyde [171–176], glyoxylic acid [177] and silicon or tin tetrachlorides [178].

SMITH [203] has described a special procedure for “distilling” reagents homogeneously onto a TLC plate. RIPPAHN [179] later started with a TLC plate that had been dipped into ninhydrin solution, laid a 0.1 mm thick terephthalate

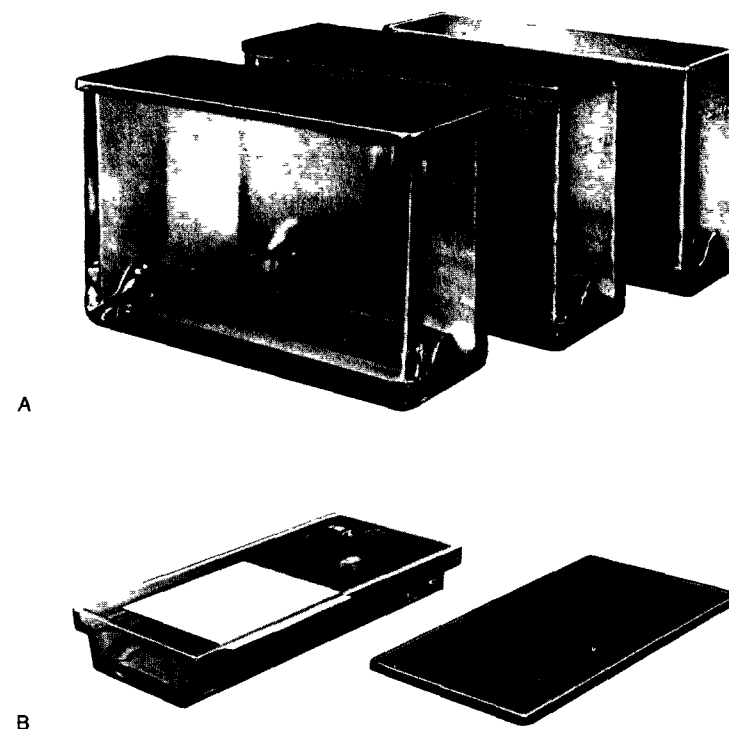


Fig. 43: Twin-trough chambers (A) and conditioning chamber (B) (CAMAG).

film frame round its edges and positioned the chromatogram bearing the amino acids to be detected so that the layer faced the “reagent plate”. This “sandwich” was then placed, reagent side down, on a hotplate. The ninhydrin then “distilled” homogeneously onto the chromatogram and stained the amino acids (see reagent Ninhydrin). It is also possible to apply *tert*-butyl hypochlorite [203], sulfuric acid and ammonia vapor [180] or acetophenone [181] in an analogous manner.

MARTINEK [182] has described the reverse procedure for relatively volatile substances (e.g. essential oil components), where the compound to be detected is “distilled” onto the reagent plate and reacts with the reagent there.

PANDEY et al. [183] employed this idea of a sandwich configuration to transfer substances from one TLC plate to another for two- or multidimensional separations.

3.2.4 Reagent in Solvent

Another method of applying a reagent homogeneously to a TLC layer is to add it to the mobile phase. A necessary precondition is that the reagent is evenly spread over the layer (the reagent must "run" with the solvent front). Double developments have frequently been described, where the first mobile phase brought about the development and the second one an improvement in zone shape and a homogeneous application of reagent to the stationary phase.

The following are amongst the reagents that have been reported as being added to the mobile phase: acids for quinine alkaloids [184], ninhydrin for amino acids [185–187], fluorescamine for biogenic amines [188], Fluorescein sodium [189], dichlorofluorescein [190], rhodamine 6G [191], ANS reagent [192] and bromine [193] have all been described as additives to mobile phases.

Dimethyl sulfoxide in the mobile phase acts as an "intrinsic detector" for certain phenols (e.g. dihydroxybenzenes) [194] on layers that have been treated with tungstate.

3.2.5 Stationary Phase as Reagent (Reagent in Adsorbent)

The exploitation of specific adsorbent properties can also lead to the same goal of homogeneous derivatization of separated substances.

Silica gel and aluminium oxide layers are highly active stationary phases with large surface areas which can, for example, — on heating — directly dehydrate, degrade and, in the presence of oxygen, oxidize substances in the layer. This effect is brought about by acidic silanol groups [93] or is based on the adsorption forces (proton acceptor or donor effects, dipole interactions etc.). The traces of iron in the adsorbent can also catalyze some reactions. In the case of testosterone and other Δ^4 -3-ketosteroids stable and quantifiable fluorescent products are formed on layers of basic aluminium oxide [176, 195].

Derivatization can also be brought about by impregnating the adsorbent layer with a suitable reagent (frequently an inorganic substance) before application of the sample solutions. This impregnation is usually performed as the layer is prepared. Here care must be taken that the reagent does not dissolve in the mobile phase and migrate towards the solvent front in the subsequent development. Such transport towards the solvent front is especially to be expected in the case of organic reagents. It is for this reason that organic components are mainly not employed for later derivatization but for homogeneous detection by fluorimetric processes (cf. Sec. 2.2.2). Examples of inorganic reagents in the adsorbent are listed in Table 19.

Table 19 Derivatization by inorganic reagents incorporated in the layer

Reagent	Substance detected, matrix	Reaction conditions, remarks	Reference
Ammonium sulfate	triglycerides, serum lipids	25–85 min at 150°C, yields fluorescent derivatives	[197]
Ammonium sulfate	phosphatidyl glycerol derivatives	charring on heating	[198]
Ammonium sulfate	phosphatidyl glycerol, sphingomyelin	10 min at 280°C densitometric in situ quantitation	[199]
Ammonium sulfate	detergents	SIL G 25 detergent plate (MACHERY-NAGEL)	[200, 201]
Ammonium sulfate	lipids	charring on heating	[202]
Ammonium sulfate	triolein, oleic acid, androsten-3,17-dione, xanthonic acid, cholesterol-propionate, N-methylphenylalanine, D-glucose	fluorescence after heating to 150–180°C, exposure to <i>tert</i> -butyl hypochlorite	[203]
Ammonium monovanadate	organic nitrogen compounds	TLC plates prepared with 2% ammonium monovanadate solution	[204]
Aluminium oxide	deoxynivalenol in wheat	7 min at 120°C, yields a fluorescent derivative under UV light ($\lambda = 365$ nm)	[193, 196]
Silver nitrate	1,2- and 1,4-dihydroxybenzene	oxidation to corresponding benzoquinones	[208]
Silver nitrate + phosphotungstic acid + cobalt nitrate	triglycerides, saturated and monounsaturated alcohols	heat to 230 to 250°C	[205, 206]
Phosphomolybdic acid	essential oil components	stabilization of the silver nitrate-impregnated adsorbent layer	[207]
Zirconium(IV) oxychloride	estrone, 17- β -estradiol and estriol, plasma lipids	heat to 150 to 180°C for 5 min. Fluorescent zones are produced — sometimes only after heating for longer period	[178]

3.2.6 Sequences of Spraying or Dipping

It is desirable, in some cases, to apply different reagents to the developed chromatograms consecutively — e.g. with intermediate drying, heating or evaluation after each reagent application. It is possible in this way to detect and identify quite different substances and substance classes on the chromatogram, particularly in the case of complex mixtures of substances. Such series application of reagents increases the selectivity of the process and allows better differentiation of substances and groups of substances.

Series application of reagents has been particularly employed in toxicological analyses in cases of intoxication or drug abuse [277–279]. However, it can be a disadvantage in the use of reagents in series that the detection evidence produced by one reagent may be disturbed by the application of a later one. It is, therefore, necessary to carefully examine and document the chromatogram after the application of each reagent. Some typical reagent series are discussed in a later Volume.

3.2.7 Processing the Chromatogram

Fortunately in recent years there has been more consideration of the direct quantitative analysis rather than concentrating just on the question of substance-specific detection and increasing detection sensitivity. So that the reagents are no longer regarded merely as “visualizers” for chromatogram zones (qualitative evidence) but are used deliberately as tools to perform reproducible, stoichiometric reactions, which are a suitable basis for quantitative analyses. This means that it is necessary to pay greater attention to the processing of the chromatogram. This begins with the drying of the chromatogram after development and the homogeneous application of the reagent and continues with the reaction and stabilization of the products of reaction.

It is known that not all reactions proceed in the same manner on all adsorbent layers because the material in the layer may promote or retard the reaction. Thus, GÄNSHIRT [209] was able to show that caffeine and codeine phosphate could be detected on aluminium oxide by chlorination and treatment with benzidine, but that there was no reaction with the same reagent on silica gel. Again the detection of amino acids and peptides by ninhydrin is more sensitive on pure cellulose than it is on layers containing fluorescence indicators [210]. The NBP reagent (*q.v.*) cannot be employed on Nano-Sil-C₁₈-100-UV₂₅₄ plates because the whole of the plate background becomes colored.

The reasons for the above phenomena are to be found in differing configurations of hydrogen bonds, the effect of pH, differences in the structures of fluorescence indicators and binders and differences in surface area. For example, silica gel 60 possesses a surface area of 500 m²/g [211] while that of Si 50 000 lies below 5 m²/g [212].

In the case of substances whose structures are pH-dependent (e.g. phenols, carboxylic and sulfonic acids, amines etc.) it is possible to produce fluorescences or make them disappear by the deliberate manipulation of the pH [213] (Table 20). Shifts of the positions of the absorption and emission bands have also been reported. This is particularly to be observed in the case of modified silica gels, some of which are markedly acidic or basic in reaction (Table 25).

Table 20: Some substances, that change the color of their fluorescence at moderate pH range [214].

Compound	Color change		pH range
	from	to	
Fluorescein	weak yellow	yellow	4.0... 5.0
2',7'-dichlorofluorescein	weak yellow	yellow	4.0... 6.0
Resorufin	colorless	orange	4.0... 6.0
Acridine	green	blue	4.5... 6.0
Quinine	blue	violet	5.9... 6.1
Thioflavine	colorless	green	6.5... 7.6
Umbelliferone	orange	blue	6.5... 8.0
4-Methyl umbelliferone	weak blue	blue	6.5... 8.0
2-Naphthol	weak blue	blue	7.0... 8.5
Morin	weak green	green	7.0... 8.5
1-Naphthol	colorless	blue green	7.0... 9.0
Cumarin	weak green	green	8.0... 9.5
Acridine orange	weak yellow-green	yellow	8.0... 10.0

3.2.7.1 Drying the Chromatogram

After a chromatogram has been developed the TLC plate is removed from the developing chamber and the status quo is fixed by removing the mobile phase remaining in the layer as quickly as possible. This is properly performed in the fume cupboard so as not to contaminate the laboratory with solvent fumes. If possible the TLC plate should be laid horizontally because then as the mobile phase evaporates the separated substances will migrate evenly to the surface where they can be the more readily detected. A fan or hair dryer (hot or cold air stream)

is often employed to increase the gradient of the solvent vapor over the surface of the layer. It must, however, be checked whether this evaporation affects the substances, for

- essential oil components may evaporate and produce mists in the direction of the air stream, some of which may redeposit on the active layers producing single-sided, fuzzy zone boundaries;
- oxygen-sensitive components may be destroyed by drying at elevated temperatures;
- particles of dust from the laboratory air can deposit on the chromatograms and may possibly affect the following analysis;
- chemical vapors are transported in the air stream onto the activated layers.

For these reasons many research groups prefer to dry the chromatograms in a vacuum desiccator with protection from light. Depending on the mobile phase employed phosphorus pentoxide, potassium hydroxide pellets or sulfuric acid can be placed on the base of the desiccator, to absorb traces of water, acid or base present in the mobile phase.

A further but also more time-consuming advance is to employ the AMD system (CAMAG). Here the mobile phase and mobile phase vapor is sucked out of the chamber and from the TLC plate after every development. This reduces to a minimum the contamination of the place of work with possibly injurious solvent vapors.

3.2.7.2 Effect of Heating after Application of Reagent

Almost all chemical reactions proceed more rapidly at elevated temperatures than in the cold and so it is recommended that the chromatogram treated with reagent be heated. Irradiation with UV light (high-pressure mercury lamp, Fig. 44) also promotes reaction. Heating to 100 to 120°C for 5 to 10 min is often sufficient to ensure complete reaction. However, the pyrolysis of organic compounds requires temperatures of 200 to 250°C, which would probably result in the whole background of the plate being darkened, because the binder in the layer also chars. The evaporation of volatile reagent during heating is avoided by laying on a covering plate [215].

Since it still is not a simple matter to heat a TLC plate really homogeneously there is a danger of reaction inhomogeneities on the plate. The usual types of apparatus employed for heat production and transfer are drying cupboards, hotplates and IR sources. The success obtained using microwaves has been modest up to now.

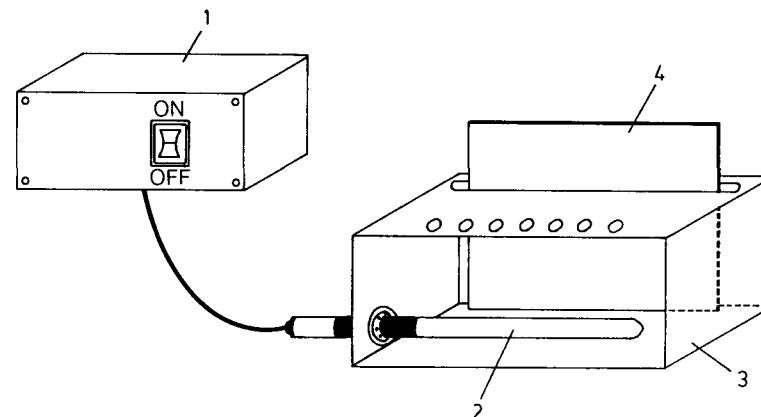


Fig. 44: Apparatus for irradiation with UV light. — 1 excitation apparatus (HERAEUS, Hanau; OSRAM StE 501), 2 UV lamp (TNN 15-31001721), 3 housing, 4 TLC plate.

Drying Cupboards

Drying cupboards are often employed for heating chromatograms after they have been treated with reagents. The TLC plate should not be placed directly on the perforated shelf of the cupboard, since the heat transfer rate is appreciably greater where contact is made with metal than where the contact is with air and the pattern of the holes on the shelf would become visible on the chromatogram. There is, thus, the danger that the reaction of the substances would be dependent on the position on the chromatogram and, hence, the reproducibility of direct quantitative analysis would suffer.

It has been found to be better to set the TLC plate down vertically on the bottom of the drying cupboard and to lay the glass upper edge against the wall of the cupboard. In cupboards with air circulation the chromatogram should be "suspended" in the air and only supported at the corners in an analogous manner to the column in a gas chromatograph oven. It has been suggested that homogeneous heating be achieved by attaching the TLC plate to a turntable which is continuously rotated inside the oven. The results obtained with such a "carousel" are reported to be good, but the method has not come into general use at least until now.

Hotplates

Hotplates (Fig. 45) are coming into increasing use for heating chromatograms. They have the advantage that it is possible to follow the reaction visually and the

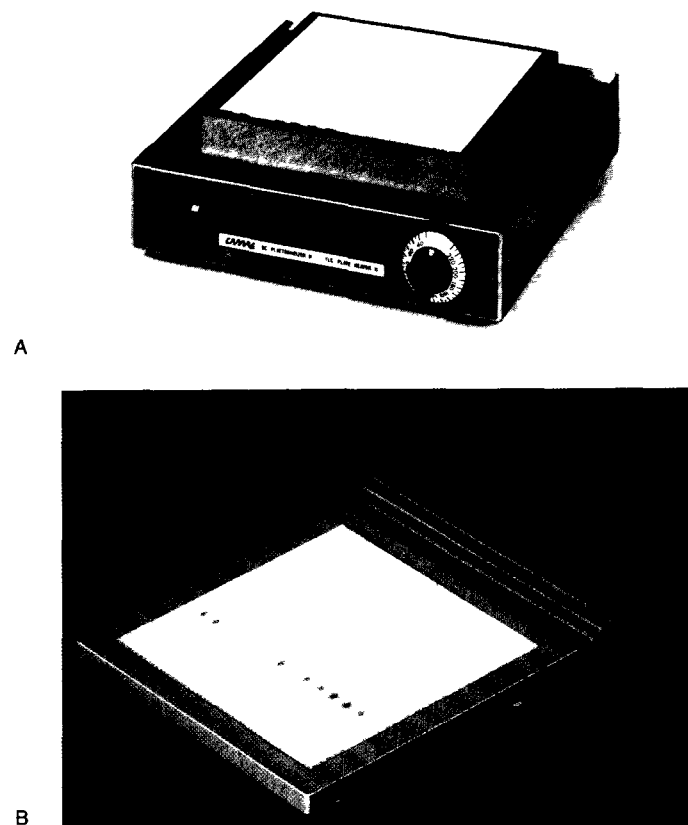


Fig. 45: Commercial hot plates A) CAMAG, B) DESAGA

reagent vapors can escape directly (fume cupboard!). The TLC plate is removed as soon as the optimal color development is produced.

Hotplates can normally be regulated over a temperature range of 30 to 190 °C. The temperature set should be maintained to an accuracy of within 2 °C, but this is not usually achieved in practice. Figure 46 shows the results of a representative range of measurements, where the effective temperature was determined as a function of the temperature setting. The temperatures were determined by means of 25 thermal sensors, which had previously been checked against each other. They were distributed over the hotplate according to the pattern shown in Figure 46B.

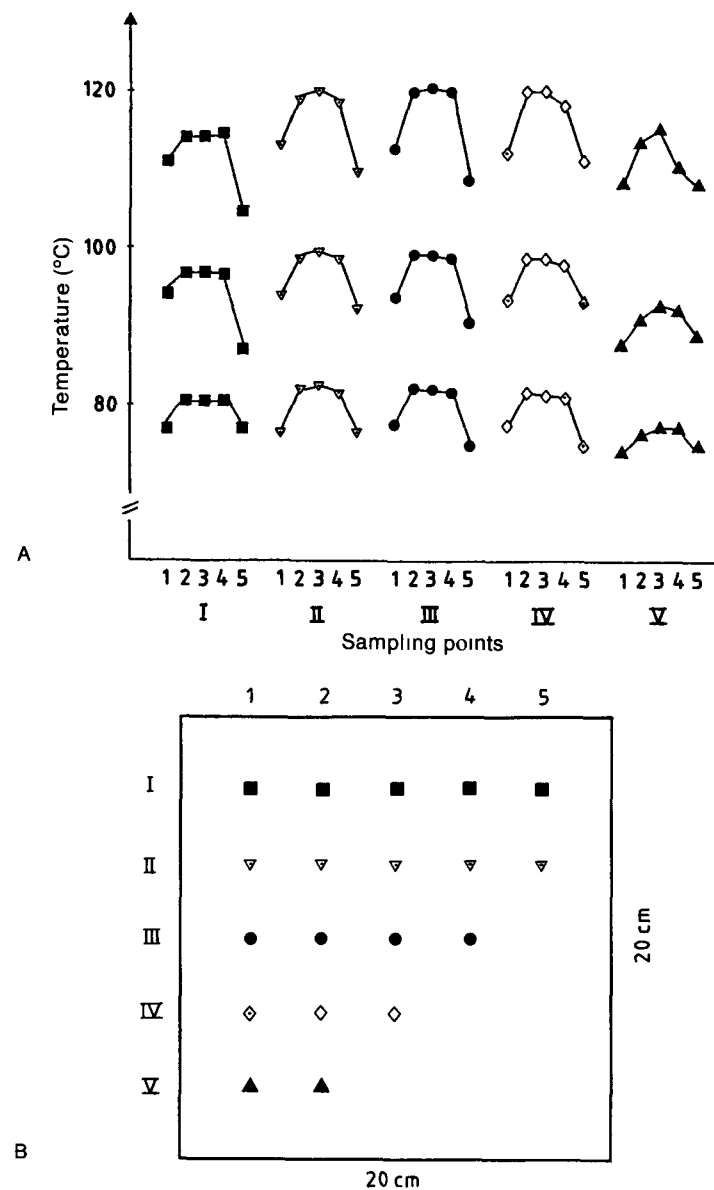


Fig. 46: Evaluation of the suitability of a hot plate for TLC by determination of the temperature distribution, A) results of 25 thermal elements at temperature settings of 80 °C, 100 °C and 120 °C, B) pattern of measuring points in five tracks (I–V) each with five measuring points

At low temperatures the average temperatures calculated from the individual measurements corresponded to the temperature setting. They were appreciably lower at higher temperatures and it was found that the temperature setting corresponded to the highest temperature that could be reached in the individual measurements. It was also evident that the edge of the hotplate was colder than the middle, i.e. the effective measured temperature was not the same everywhere over the surface of the hotplate; a homogeneous temperature distribution is most likely to be found in the center of the plate.

In the derivatization of sugars with aniline-diphenylamine reagent for example, this leads to unsatisfactory irregular coloration. The standard deviation for the method deteriorates from 2 to 3% to 5 to 8%. For this reason color reactions should be avoided for direct quantitation if it is possible to scan in the UV range without derivatization.

IR Sources

Infrared sources are sometimes employed to heat thin-layer chromatograms. The chromatogram is laid on an insulating foil and irradiated from above at a distance of 10 to 20 cm by quartz spirals which are heated to 800°C. Contact difficulties with the base naturally do not play any rôle here. But there is usually no regulation of the rate of heating so that such lamps can only be satisfactorily employed for pyrolysis investigations. They are insensitive to acid vapors and other aggressive reagents.

Microwave Apparatus

The methods of heating TLC/HPTLC plates described above depend on thermal conduction, convection or radiation. Microwave heating involves a special form

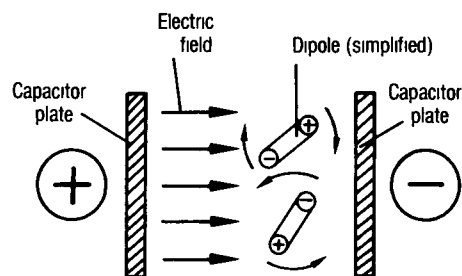


Fig. 47: Orientation of dipoles in the electric field [220].

of dielectric heating [217]. Here frictional heat is produced in a nonconducting or only slightly conducting body by a high frequency electromagnetic field. This frictional heating is a result of the fact that molecules with an intrinsic or induced (in the case of adsorbed molecules) dipole structure continually seek to align themselves to the alternating field (Fig. 47). Table 21 lists the dipole moments μ of some substances. The more symmetrically the charge is distributed in the molecule the smaller is the dipole moment.

Table 21: Dipole moments of some substances.

Substance	Dipole moment μ [D]*	Substance	Dipole moment μ [D]*
Chlorine gas, carbon dioxide	0	D-Ribose	5.1
Ammonia	1.48	D-Galactose	5.3
Sulfur dioxide	1.61	Glycine	13.3
Methanol	1.67	Alanine	17.5
Water	1.84	Myoglobin (whale)	155
Acetone	3.7	Oxyhemoglobin, carboxyhemoglobin	400
D-Glucose	4.7		

* 1 D (DEBYE) = $3.33 \cdot 10^{-30}$ cm

The frequency of microwave radiation lies between that of IR radiation and high frequency radio waves and the boundaries between these regions are not fixed [221]. The microwaves are generated in a transmitter (magnetron) which possesses a "stalk" which penetrates like a radio antenna into a hollow energy guide (Fig. 48). This leads the electromagnetic waves into the reaction chamber (power about

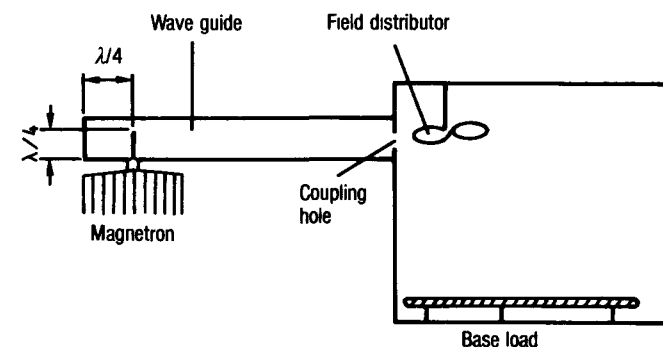


Fig. 48: Schematic representation of a microwave apparatus [222].

600 W) At the entrance an aperture focuses the microwaves on a rotating metal propeller (wave mixer), which distributes the radiation throughout the reaction chamber and prevents the setting up of standing waves which would result in uneven heating of the TLC plates [217, 223]

Modern apparatus are equipped with a rotating table to accommodate the TLC plate above the base of the reaction chamber. This means that microwaves can penetrate the TLC plate from below through the glass plate or plastic film. Aluminium foil backings are not suitable! They reflect the radiation and high potentials are built up between the aluminium foil and the wall of the reaction chamber, these result in electrical discharges.

So long as water is present in the adsorbent layer, the temperature does not rise above 100°C. However, microwave heaters will even perform pyrolyses when this has evaporated.

It follows from the depth of penetration of the microwaves, which is calculated

from the formula $d = \frac{c}{f \sqrt{\epsilon}}$ (f = frequency, ϵ = dielectric constant of the adsorbent, c = velocity of propagation of electromagnetic waves in vacuo) [225], that

practically the same temperature is reached at every depth in the TLC layer during microwave heating. This is an advantage compared with hotplates. In addition, the reaction is completed more rapidly (after 1–2 min) than in the drying cupboard. In spite of these advantages microwave heaters have found scarcely any application in TLC analysis.

3.2.7.3 Stabilization of Developed Zones

Treatment of the chromatogram with a reagent results in the production of colored or fluorescent chromatogram zones, which are used to evaluate the success of the separation and for quantitative analysis. For this purpose it is necessary that the color or fluorescence intensities remain stable for about 30 minutes.

There are no general recommendations applicable to the stabilization of the color of *colored chromatogram zones* apart from that of storing the chromatograms in an atmosphere of nitrogen and protected from light until they are evaluated. There are naturally other color stabilization methods which are applicable. Well known is the addition of cadmium or copper salts in the case of the ninhydrin reagent ($q \nu$) and spraying with sodium nitrite solution after the van URK reaction for lysergic acid derivatives [226]. The blue color of tryptamine after reaction with 2,6-dibromoquinone-4-chloroimide ($q \nu$) can also be stabilized, this time by exposing to ammonia vapor or spraying with ammonia solution [103].

In the case of *fluorescent chromatogram zones* there is also, in addition to storage of the chromatogram in the absence of light and oxygen mentioned above (Fig. 49 and 50), another method of stabilization, namely treatment of the chromatogram with viscous lipophilic or hydrophilic agents. These evidently reduce the ease with which parts of the molecules rotate and keep out the laboratory air. Singlet oxygen, which is the primary agent in the oxidative degradation of substances, is not

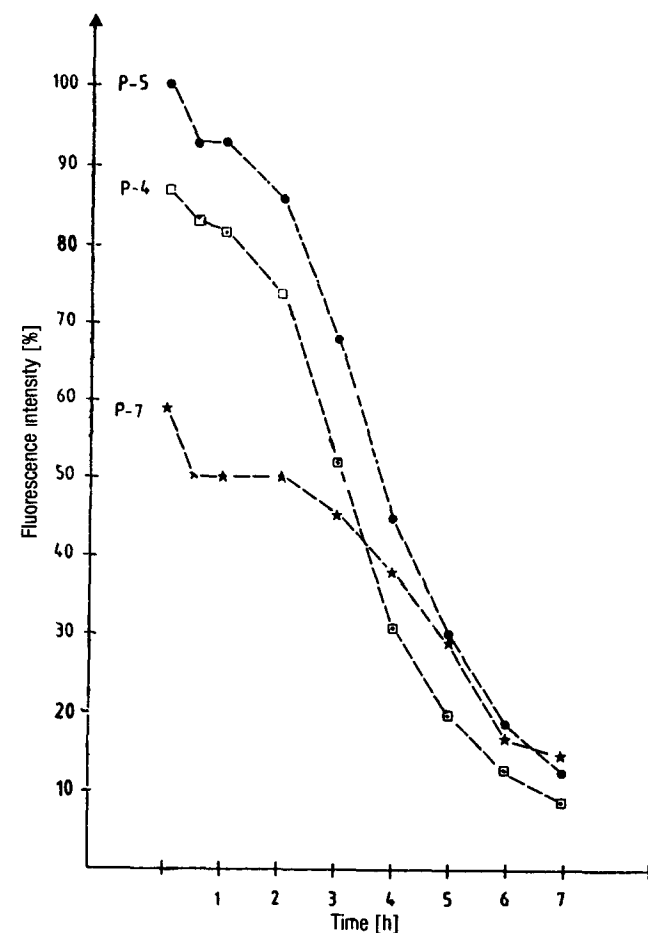


Fig. 49: Reduction in the fluorescence of coproporphyrin (P-4), pentaporphyrin (P-5) and heptaporphyrin (P-7) as a function of time (storage of chromatogram in the air and in daylight)

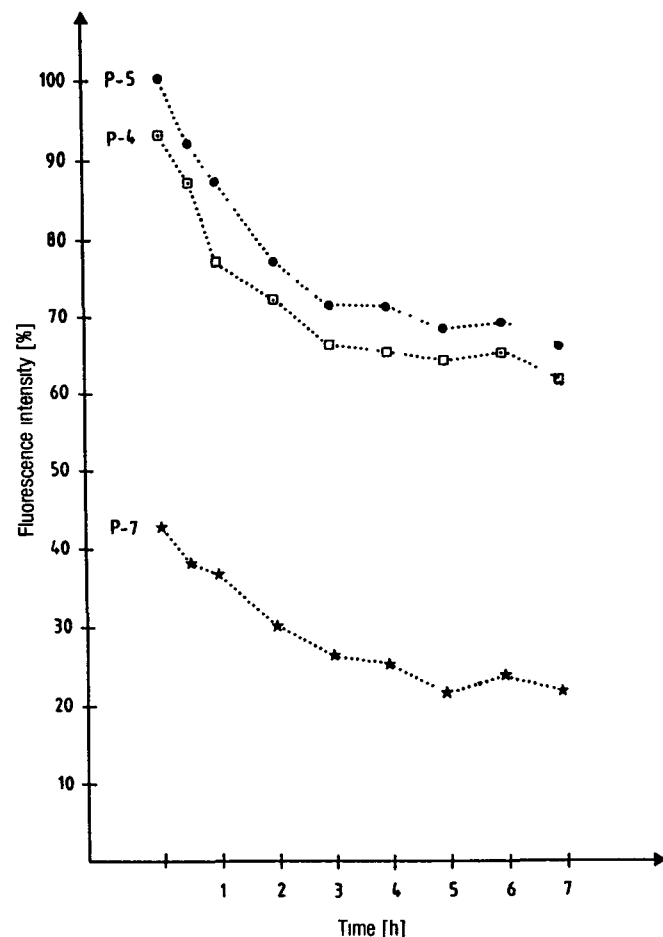


Fig. 50: Reduction in the fluorescence of coproporphyrin (P-4), pentaporphyrin (P-5) and heptaporphyrin (P-7) as a function of time (storage of chromatogram in the dark)

excluded [227], but it is converted to less aggressive triplet oxygen during transport through the lipophilic phase.

In 1967 spraying with a solution of paraffin wax allowed the recording of the fluorescence spectrum of anthracene directly on the TLC plate without any difficulties [228]. HELLMANN too was able to stabilize emissions by the addition of 2% paraffin to the solvent [229]. Low concentrations evidently serve primarily to *stabilize the fluorescence* – this “stabilization concentration” extends up to ca

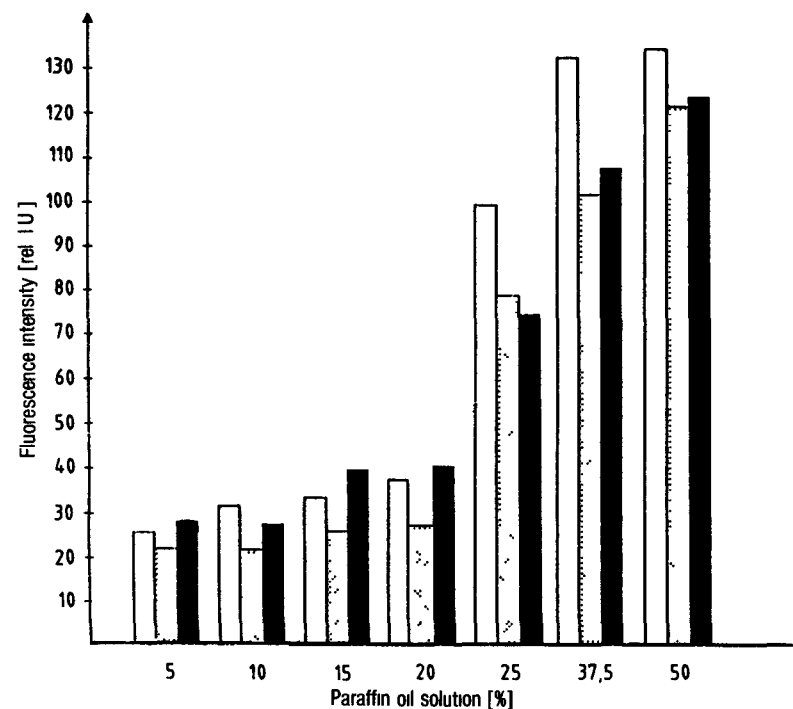


Fig. 51: Fluorescence intensities of porphyrins as a function of the concentration of the paraffin oil dipping solution: ■ mesoporphyrin, ▨ coproporphyrin, □ pentaporphyrin

20% in the case of porphyrins (Fig. 51). If the chromatograms are dipped in more concentrated solutions the fluorescence yield suddenly jumps. It is not clear whether micelle formation plays a rôle in this intensification.

Figure 49 shows that porphyrins are decomposed in the layer within a few hours if no special measures are taken, but that they can be stabilized for more than 24 hours if the layers are dipped in 50% paraffin solution and stored in the dark. This was true of all six porphyrins investigated (Fig. 52). Quantitation should not be undertaken less than an hour after dipping the chromatograms, because it takes so long for the fluorescence emission to stabilize [230].

Similar fluorescence-stabilization has been reported for polyethylene glycol 4000 by WINTERSTEIGER [291].

FUNK et al. [231] have demonstrated that the sensitivity of the analysis can be extended down into the femtogram range for the determination of selenium in water and biological matrices (Fig. 53).

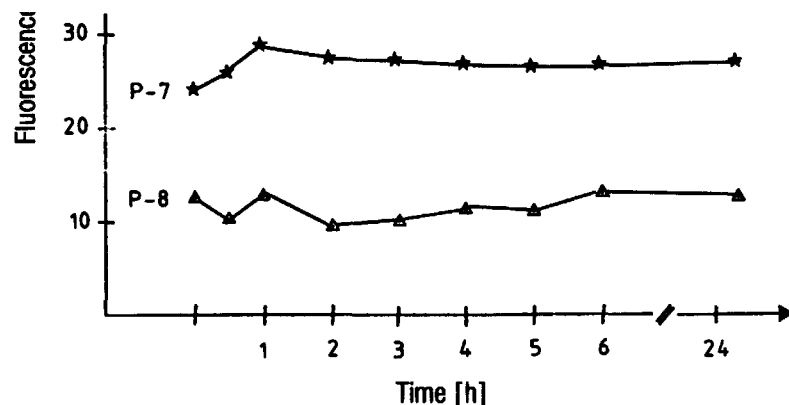


Fig. 52: Fluorescence intensity of porphyrin chromatogram zones as a function of time after dipping in 50% liquid paraffin solution and storage in darkness. — P-2: mesoporphyrin, P-4: coproporphyrin, P-5: pentaporphyrin, P-6: hexaporphyrin, P-7: heptaporphyrin, P-8: uroporphyrin.

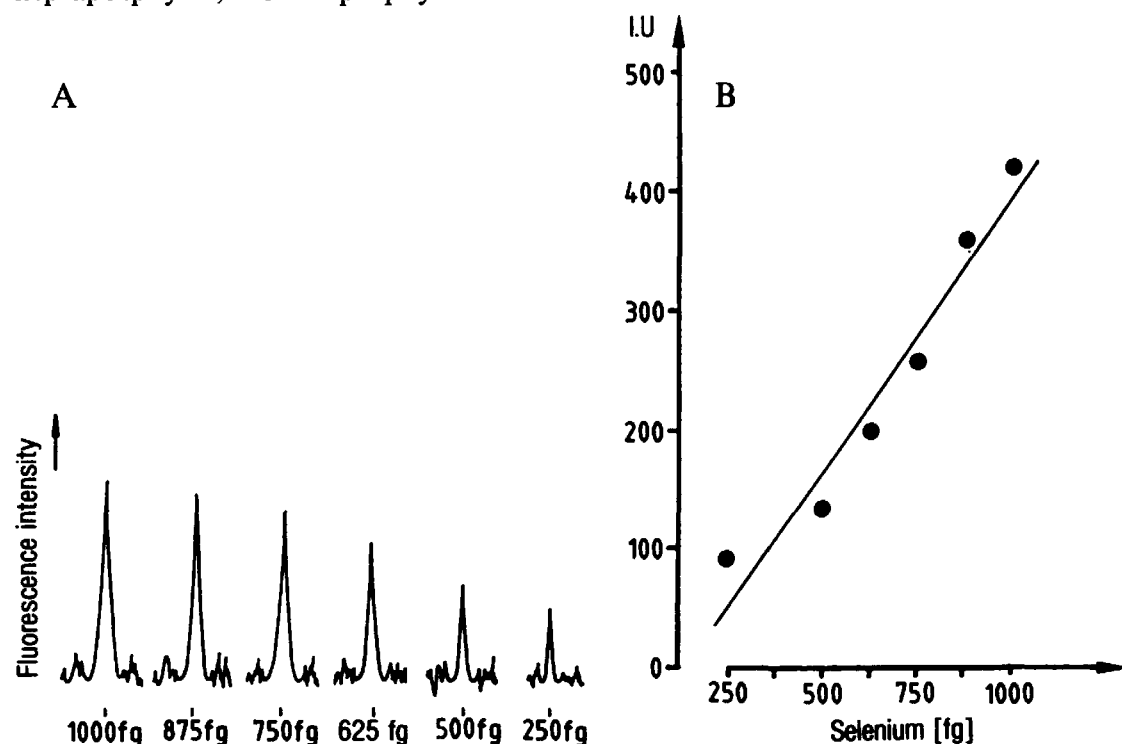


Fig. 53: Fluorescence scan of femtogram quantities of 2,1,3-naphthoselenodiazole (A) and associated calibration curve (B).

Table 22: Lipophilic fluorescence intensifiers

Fluorescence intensifier	Substances	Sensitization
Isooctane	porphyrin methyl esters	reactive fluorescence
Dodecane	polycyclic aromatic hydrocarbons	2 to 10
Palmitic and stearic acids	aflatoxins B ₁ and B ₂	15 to 20
Kerosine	porphyrins	approx. 10
Fomblin Y-Vac	polycyclic aromatic hydrocarbons	up to 10
Fomblin H-Vac	1-aminopyrene	stable, high
Liquid paraffin	benzo(a)pyrene	35 to 40
Liquid paraffin	aflatoxins	3 to 5
Liquid paraffin	aflatoxin B ₁	2.5 to 3
Liquid paraffin	aflatoxins, sterigmatocystine	10 to 15

Table 22 (Continued)

Fluorescence intensifier	Substances	Sensitivity increase/stabilization	Remarks/references
Liquid paraffin	selenium as 2,1,3-naphthoselenodiazole	25-fold	spray solution, 67% in <i>n</i> -hexane (Fig 53 [240])
Liquid paraffin	carbamate and urea herbicides	stabilization and enhancement	spray solution, 20% in toluene, water investigation [241]
Liquid paraffin	digitalis glycosides	stabilization and enhancement	30% in chloroform [242]
Liquid paraffin	digitoxin, digoxin, methyldigoxin	enhancement	the chromatogram was coated with a film of paraffin liquid [243]
Liquid paraffin	luteoskyrin, rugulosin	stabilization	spray solution, 50% in toluene—ethyl acetate—formic acid (16 + 4 + 1) [244]
Liquid paraffin	Δ^4 -3-ketosteroids	10-fold	dipping solution, 33% in <i>n</i> -hexane [10]
Liquid paraffin	Δ^4 -3-ketosteroids (testosterone isonicotinic hydrazone, testosterone dansyl hydrazone)	> 10-fold	dipping solution, 33% in <i>n</i> -hexane [232]
Liquid paraffin	cholesterol, coprostanone, coprostanol etc	2 to 8-fold	dipping solution, 33% in <i>n</i> -hexane [246]
Liquid paraffin	estriol as dansyl derivative	10-fold	dipping solution, 67% in <i>n</i> -hexane [247]
Liquid paraffin	cortisol as dansyl hydrazone	10-fold	dipping solution, 67% in <i>n</i> -hexane, serum investigations [248, 249]
Liquid paraffin	dansylamides	10-fold, stabilization > 10 h	spray solution, 67% in <i>n</i> -hexane [245]
Liquid paraffin	amiloride	80% increase	dipping solution, 33% in cyclohexane, human plasma investigation [250]
Liquid paraffin	fluphenazine	enhancement	dipping solution, 5% in toluene, plasma investigation [251]

Table 22 (Continued)

Fluorescence intensifier	Substances	Sensitivity increase/stabilization	Remarks/references
Liquid paraffin	piroxicam	enhancement	dipping solution, 10% in <i>n</i> -pentane, urine, tissue and plasma investigations [252]
Liquid paraffin	gentamycins, netilmicin	50 to 65% enhancement	dipping solution, 15% in <i>n</i> -hexane [253]
Liquid paraffin	gentamycins	stabilization	dipping solution, 33% in <i>n</i> -hexane [254]
Liquid paraffin	morphine as dansyl derivative	stabilization	dipping solution, 20% in <i>n</i> -hexane [291]
Liquid paraffin—triethanolamine	carbamazepine	30-fold	chloroform—liquid paraffin—triethanolamine (60 + 10 + 10) [255, 256]
Liquid paraffin—triethanolamine	cis/trans-diethylstilbestrol, ethynylestradiol	3-fold	chloroform—liquid paraffin—triethanolamine (60 + 10 + 10) [257]
Liquid paraffin—triethanolamine	vitamin B ₁	2-fold	chloroform—liquid paraffin—triethanolamine (60 + 10 + 10) [258]
Silicone DC 200	sterigmatocystine	10-fold	18% in diethyl ether, cheese investigation [259]

Hydrophilic liquids can also cause stabilization and amplification of fluorescence. Thus, DUNPHY et al. employed water or ethanol vapor to intensify the emissions of their chromatograms after treatment with 2',7'-dichlorofluorescein [260]. Some groups of workers have pointed out that the layer material itself can affect the yield of fluorescent energy [261–263]. Thus, polyamide and cellulose layers were employed in addition to silica gel ones [245]. The fluorescence yield was generally increased by a factor of 5 to 10 [264], but the increase can reach 100-fold [234, 265].

Some examples of fluorescence amplification with the aid of hydrophilic liquids are listed in Table 23.

Table 23 Hydrophilic fluorescence intensifiers and their fields of application

Fluorescence intensifier	Substances	Sensitivity increase/stabilization	Remarks/references
Ethylene glycol	furosemide and its metabolites	no information	10% citric acid in ethylene glycol – water (1 + 1), plasma investigations [266]
2-Ethoxyethanol	gramine	stabilization	6% in mobile phase [267]
Glycerol	ethoxyquin, dansyl amides	20-fold	spray solution, 33% in methanol [292] or 50% ethanol [245]
Polyethylene glycol 400	cysteine adducts of α -, β -unsaturated aldehydes as dansyl hydrazones	stabilization and enhancement	dipping solution, 25% in chloroform [268]
Polyethylene glycol 400	compounds with alcoholic OH groups	20 to 25-fold	dipping solution, 10% in methanol [269]
Polyethylene glycol	primary amines, indole derivatives, sympathomimetics	stabilization and enhancement	dipping solution, 20% in methanol [270]
Polyethylene glycol 4000	alcohols, amines	stabilization and enhancement	spray solution, 50% in methanol, best results on silica gel [271]
Polyethylene glycol 4000	flavonoide glycosides	no information	dipping solution, 5% in ethanol, phytochemical investigations [273]
Polyethylene glycol 4000	silymarin	enhancement	5% in ethanol, optimum for fluorescence after 24 h [274]
Polyethylene glycol 4000	primary, secondary, tertiary alcohols as anthracene-urethane derivatives	stabilization and enhancement	saturated dipping solution in methanol [275]
Polyethylene glycol 4000	compounds with alcoholic OH groups	20 to 25-fold	dipping solution, 10% in methanol [269]
Polyethylene glycol 4000	cetanol after reaction with 8-bromomethylbenzo-d-pyrido(1,2-a)pyrimidin-6-one	stabilization > 15 d	dipping solution, 10% in chloroform [291]

Table 23 (Continued)

Fluorescence intensifier	Substances	Sensitivity increase/stabilization	Remarks/references
Triethylamine	phenylurea and N-phenylcarbamate pesticides	stabilization and enhancement	spray solution, 10% in dichloromethane, 15 min delay before analysis [41]
Triethylamine	aminoglycoside antibiotics	stabilization	spray solution, 10% in dichloromethane, investigation of solutions for injection [276, 278]
Triethylamine	amino acids as fluorescamine derivatives	stabilization	spray solution, 10% in dichloromethane, employed before and after reaction with fluorescamine [280]
Monoethanolamine	dansyl amides	10-fold	spray solution [245, 272]
Triethanolamine	dansyl amino acids, dansyl amides	enhancement	spray solution, 20% in 2-propanol, fluorimetric evaluation after 16 h storage in vacuo [281]
Triethanolamine	gramine	enhancement	20% in water, sodium hydroxide solution ($c = 1$ mol/l) or 20% monoethanolamine solution can also be employed [267]
Triethanolamine	ephedrine, effortil and estriol as dansyl derivatives	stabilization > 100 min	20% in 2-propanol [282]
Triethanolamine	spermine, spermidine	no information	spray solution, 20% in 2-propanol, fluorimetric evaluation after 16 h storage in vacuo [283]
Triethanolamine	N-nitrosamines	no information	spray solution, 10% in dichloromethane [284]
Triethanolamine	thiourea	enhancement	spray solution, 20% in 2-propanol [285]
Triethanolamine	carbamate and urea herbicides as dansyl derivatives	stabilization	20% in 2-propanol, soil investigations [286, 287]
Triethanolamine	matacil, zectran	stabilization	spray solution, 20% in 2-propanol [288]

Table 23: (Continued)

Fluorescence intensifier	Substances	Sensitivity increase/stabilization	Remarks/references
Triethanolamine	propham, chloroprotham, swep, linurone, maloron	stabilization and enhancement	spray solution, 20% in 2-propanol, water investigations [241]
Triton X-100	dansyl amides and amino acids	30 to 110-fold	spray solution, 20% in chloroform [265, 290]
Triton X-100	ethoxyquin (antioxidant in spices)	> 200-fold, stabilization > 15 h	spray solution, 33% in benzene; the fluorescence of aflatoxin B ₁ is reduced by 10 to 15% [292].
Triton X-100	polycyclic aromatic hydrocarbons	10-fold	1% solution in <i>n</i> -hexane; optimal emission after 60 min; 10% zone enlargement [234]
Triton X-100	testosterone dansyl hydrazone	> 25-fold	dipping solution, 20% in chloroform [232]
Triton X-100	selenium as 2,1,3-naphthoselenodiazole	90-fold	dipping solution, 20% in chloroform (Fig. 53 [231])
Sodium dodecylsulfate, cetyltrimethylammonium chloride, sodium cholate, β -cyclodextrin	dansylated amino acids and polycyclic aromatic hydrocarbons	> 45-fold	1% in water; the greatest enhancement of fluorescence is that of sodium cholate on pyrene [263]
Diocetyl sulfosuccinate	codeine, morphine, monoacetyl-morphine, heroin	stabilization	dipping solution, 20% in ethanol [94]

Although there is ample experimental evidence confirming the stabilization and amplification of fluorescence by means of viscous lipophilic and hydrophilic liquids there is as yet no convincing physicochemical explanation of the phenomenon. Wetting phenomena and pH changes could also play a rôle alongside solubilization phenomena and micelle formation in the liquid film. As a nonionogenic detergent Triton X-100 possesses wetting properties, so that here a surface-active effect can be brought into the discussion. The amines that have been employed certainly displace the pH. Further investigations are required to clarify the phenomenon.

3.3 Biological-Physiological Methods of Detection

Just like the physical and microchemical methods of detection, the indirect, biological-physiological detection procedures are very selective when applied to thin-layer chromatography. Here it is not chemical functional groups or particular physical properties that are selectively detected but effects on highly sensitive "biodefectors". The following detection techniques have been employed:

- *Manual transfer* of the chromatographically separated substance to the "detector". These include, for example, the detection of antibioticly active substances, plant and animal hormones, mycotoxins, insecticides, spice and bitter principles and alkaloids. The frequency distribution of their employment is shown in Figure 54 [295].
- *Bioautographic determinations*, where test organisms, tissue homogenates or cell organelles are applied in agar or gelatine solution as detectors directly on the surface of the developed chromatogram. The detection of antibiotics, fungicides, saponins, vitamins etc. have been described using this method.
- *Reprint methods* where the developed dried chromatogram is laid on the prepared agar layer "detector" with the exclusion of air bubbles. In this and in the

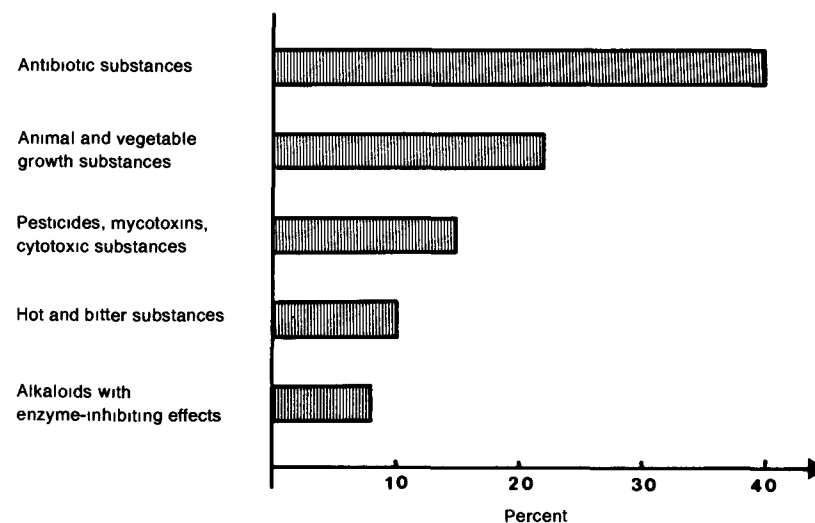


Fig. 54: Fields of application and frequency distribution of biological-physiological detection methods.

previous method the "active agents" diffuse out of the TLC layer into the 1 to 4 mm thick agar detector layer and there promote or inhibit the growth of the indicator organism during the period of incubation

- *Enzymatic determinations of the detection limit* where the chromatograms are first sprayed with an enzyme solution. Then after appropriate incubation the enzymatically altered components are detected by reaction with a suitable reagent

These methods are naturally subject to the degree of variation usual in biology and a degree of manual dexterity is often required. Their employment in TLC is, nevertheless, justified because

- these methods are highly specific (Fig. 36),
- inactive accompanying substances do not interfere,
- the detection limits of the compounds are sometimes so low that it is scarcely possible to obtain better results by the classical methods of physical chemistry without resorting to enrichment

These topics will be treated in depth in Volume 3

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4 Documentation and Hints for Chromatography Experts

Every chromatographic investigation begins with the preparation of the sample and the chromatographic system. This is followed by the crux of the separation process (development of the chromatogram) which is in turn followed by the visualization of the separated substances and the preservation of the chromatogram and finally by the analysis of the results.

Each of these steps must be so documented that it can always be repeated simply from the protocol. The most important steps will be discussed below, paying special attention to the processing of the chromatogram after development.

4.1 Preparations for Chromatography

A description of the preparatory steps before chromatography is performed from an integral part of the complete documentation. A record is necessary of the

- sampling process,
- sample storage (cool room, protection from light, inert gas atmosphere etc.),
- comminution of the sample (sieve size etc.) and
- sample preparation (extraction, distillation, sublimation etc.).

In addition, information must be provided concerning the enrichment and clean up of the sample. If possible the sample solution prepared should be adjusted to a particular concentration, so that the application of the chosen volume gives a preliminary idea of the amounts in the chromatogram produced.

Particular pieces of apparatus and chemicals and solvents of exactly defined quality were employed in the examples that follow and it is necessary to define them precisely. Manufacturers' names have been given when necessary as defining a quality criterion. The purity of solvents and chemicals is a particularly important point.

4.1.1 Solvent Quality

The higher the demands made on the analysis the higher must be the quality of the solvents employed. Since the substances are present in dissolved form during

most stages of the analysis it must, for example, be known what additives have been employed to stabilize sensitive solvents (Tab. 24).

Only particular solvents are suitable for certain purposes. The choice depending, for instance, on their residual water content or their acid-base nature if R_f values are to be reproduced [1, 2]. Halogen-containing solvents may not be employed for the determination of chlorinated pesticides. Similar considerations apply to PAH analyses. "Pro analysi" grades are no longer adequate for these purposes. It is true that it would be possible to manufacture universally pure solvents that were adequate for all analytical purposes, but they would then be too expensive for the final user [3, 4].

Table 24: List of some solvents and their stabilizers

Solvent	Quality	Stabilizer	Company
Chloroform	HPLC solvent	ethanol, 0.5 to 1%	BAKER
	hydrocarbon stabilized	amylene, 0.01 to 0.02%	BAKER
	GR	ethanol, 0.6 to 1.0%	MERCK
	LiChrosolv for analysis	amylene	MERCK
Dichloromethane	Chromasolv	ethanol, ca. 1%	RIEDEL-de HAËN
	HPLC solvent	amylene	RIEDEL-de HAËN
	GR	cyclohexane, 100 to 350 ppm	BAKER
	LiChrosolv for analysis	amylene, ca. 20 ppm	MERCK
Diethyl ether	Chromasolv	amylene, ca. 20 ppm	MERCK
	GR	amylene, ca. 25 ppm	RIEDEL-de HAËN
	dried	amylene, ca. 25 ppm	RIEDEL-de HAËN
	for analysis	BHT, 7 ppm	MERCK
Dioxane	Chromasolv	no information	MERCK
	GR	BHT, 5 ppm	RIEDEL-de HAËN
	LiChrosolv for analysis	ethanol, ca. 2%	RIEDEL-de HAËN
	Chromasolv	BHT, 25 ppm	MERCK
Diisopropyl ether	GR	BHT, 1.5 ppm	MERCK
	for analysis	BHT, 25 ppm	RIEDEL-de HAËN
	HPLC solvent	BHT, 1.5 ppm	RIEDEL-de HAËN
	GR	BHT, 5 ppm	MERCK
Tetrahydrofuran	Chromasolv	BHT, 5 ppm	RIEDEL-de HAËN
	HPLC solvent	BHT, 0.02 to 0.03%	BAKER
	GR	BHT	MERCK
	LiChrosolv for analysis	no information	MERCK
Chromasolv	Chromasolv	BHT, 250 ppm	RIEDEL-de HAËN
	Chromasolv	no information	RIEDEL-de HAËN

Large quantities of solvents are employed for sample preparation, in particular, and these are then concentrated down to a few milliliters. So particularly high quality materials that are as free as possible from residual water and especially free from nonvolatile or not readily volatile impurities ought to be employed here; such impurities are enriched on concentration and can lead to gross contamination. The same considerations also apply to preparative chromatography. Special solvents of particular purity are now available.

4.1.2 Choice of Stationary Phase

If sufficient knowledge is available concerning the previous history and chemical nature of the sample, then it is possible to choose the type of stationary and mobile phase according to the triangular scheme [5, 40] (Fig. 55). Silica gel and aluminium oxide are active adsorbents. They should be characterized more precisely when documenting the experiment, most simply by noting exactly the product designation and source, since every manufacturing company takes care to guarantee the properties and qualities of its products.

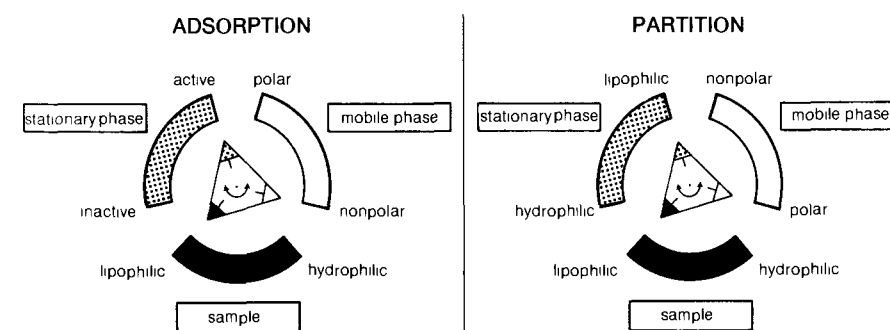


Fig. 55: Triangle scheme for the choice of phases.

Thus, for instance, 10% aqueous suspensions of the adsorbents produced by the various manufacturers are different (Table 25). It is not a matter of indifference whether Silica gel 60 or Silica gel 60 W (water-resistant) layers are employed, for the two differ appreciably both in pH and in running time and, hence, in selectivity [6].

Table 25: Summary of the pH values of some layer materials of precoated plates, determined as 10% aqueous suspensions (duplicate determination: two different TLC/HPTLC plates from the same batch).

Layer type	Company and Catalogue No.	pH	Layer type	Company and Catalogue No.	pH
Aluminium oxide 60, type E	MERCK 5713	9.7	Silica gel G 1500	SCHLEICHER & SCHÜLL	6.2
Aluminium oxide 150, type T	MERCK 5727	8.9	Silica gel G 1570	SCHLEICHER & SCHÜLL	7.5
Cellulose	MERCK 5786	6.6	Kieselguhr	MERCK 5738	7.6
Cellulose	SCHLEICHER & SCHÜLL 3793	6.8	Si 50 000	MERCK 15132	7.9
Silica gel	MACHEREY & NAGEL 809023	6.4	Silica gel 60 silanized	MERCK 5747	7.6
Silica gel	MACHEREY & NAGEL 811022	6.3	Silica gel RP-8	MERCK 13725	4.7
Silica gel 60	MERCK 5713	7.8	Silica gel RP-18	MERCK 13724	5.0*
Silica gel 60	MERCK 5721	7.6	Silica gel RP-18 W	MERCK 13124	4.4
Silica gel 60	MERCK 5628	7.7	CN phase	MERCK 16464	5.1
Silica gel 60 purest	MERCK 15552	4.7	Diol phase	MERCK 12668	3.6
Silica gel 60 with conc. zone	MERCK 11798	6.8	NH ₂ phase	MERCK 15647	9.9
Silica gel 60	RIEDEL-de HAËN 37643	7.6	Polyamide 11	MERCK 5557	6.9
Silica gel	RIEDEL-de HAËN 37601	6.4			

* Suspension well shaken, afterwards the RP-18 material floated on the surface.

Aluminium oxide is available in grades with neutral, acidic and basic reactions, which can also vary in the specific surface area and pore size. This makes the separations achieved vary and care must be taken to document precisely.

Polyamide is available commercially as polyamide-6 (based on ϵ -aminocapro-lactam, MACHEREY-NAGEL) and as polyamide-11 (based on 11-aminoundecanoic acid, MERCK). The lipophilic properties of these are different, thus altering their chromatographic selectivity.

Cellulose layers are produced from native, fibrous or microcrystalline cellulose (Avicel®). The separation behaviors of these naturally vary, because particle size (fiber length), surface, degree of polycondensation and, hence, swelling behavior are all different.

The most important thing to pay attention to in the case of *RP phases* is the chain length. It is often forgotten, however, that RP phases are available with differing degrees of surface modification and which also differ in their hydrophobicity and wettability and separation behavior (R_f values, development times). These details should, therefore, also be documented.

Differences in the materials employed for the layers can also become evident when chemical reactions are performed on them. Thus, MACHEREY-NAGEL report that the detection of amino acids and peptides by reaction with ninhydrin is less sensitive on layers containing luminescent or phosphorescent indicators compared to adsorbents which do not contain any indicator [7].

It is for this reason that the details given in the monographs are, on the one hand, obtained by reviewing the literature while, on the other hand, the "Procedure Tested" section reports the results we ourselves obtained, which are not necessarily in complete agreement with the literature reports.

The fact that the binder used in the layer can affect the reagent is shown in the monograph on 4-(4-Nitrobenzyl)pyridine reagent. It is not possible to employ this reagent on Nano-SIL C 18-100 UV₂₅₄ plates (MACHEREY-NAGEL) because the whole surface of the layer is colored bluish-violet. The corresponding water-wettable layers produced by the same manufacturer do not present any difficulties.

These few remarks should suffice to demonstrate the importance of the precise knowledge of the various layer materials and the precise documentation of their use. Such differences should also be taken into account when choosing the stationary phase so that the impression is not later produced that phase A is better or worse than phase B.

4.1.3 Prewashing the Layer

All commercially available precoated plates are manufactured with great care. But they are active layers which, on account of the numbers and structures of their pores, possess a very large internal surface area, on which water vapor and other volatile substances can condense, particularly once the packaging has been opened. In order to prevent this as far as possible the precoated plates are packed with the glass or foil side upwards.

It is possible that plasticizers or monomeric components of the packing materials (adhesives etc.) are also absorbed during storage. In order to stop such "impurities" or oligomeric components of the binder interfering with the development, the plates are often prewashed before the actual chromatography, particularly during quantitative work. This is done by developing the chromatogram one or more times with either a mixture of methanol and chloroform (1 + 1) or with the mobile phase that is to be employed later. When doing this the mobile phase should be allowed to climb appreciably above the solvent front of the subsequent chromatographic run. Acidic or basic mobile phases can cause difficulties because they are not completely removed during the subsequent activation (30 min at 110°C) in the drying cupboard and, thus, "impregnate" the stationary phase.

It is always inadvisable to activate in a stream of hot or cold air (hair drier), because laboratory air is then blown over the layer. Such details also belong in the documentation of working instructions.

4.1.4 Choice of Chamber System

There is no other facet where thin-layer chromatography reveals its paper-chromatographic ancestry more clearly than in the question of development chambers (Fig. 56). Scaled-down paper-chromatographic chambers are still used for development to this day. From the beginning these possessed a vapor space, to allow an equilibration of the whole system for partition-chromatographic separations. The organic mobile phase was placed in the upper trough after the internal space of the chamber and, hence, the paper had been saturated, via the vapor phase, with the hydrophilic lower phase on the base of the chamber.

In the case of thin-layer chromatography there is frequently no wait to establish complete equilibrium in the chamber before starting the development. The chamber is usually lined with a U-shaped piece of filter paper and tipped to each side after adding the mobile phase so that the filter paper is soaked with mobile phase and adheres to the wall of the chamber. As time goes on the mobile phase evaporates from the paper and would eventually saturate the inside of the chamber.

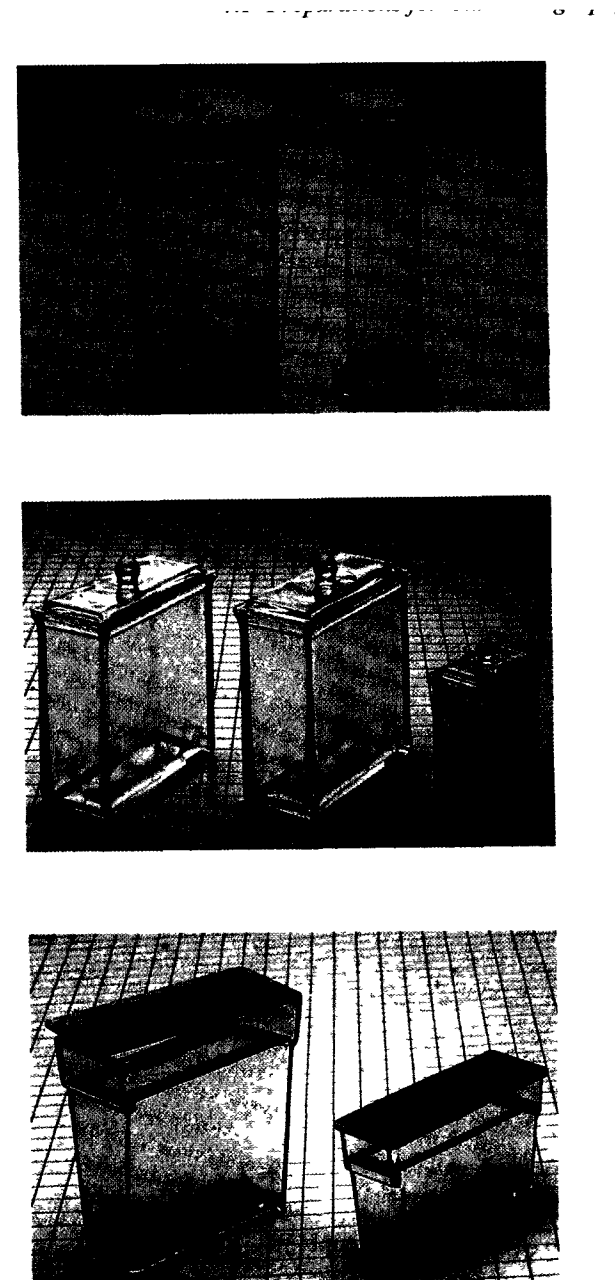


Fig. 56: Commonly used trough chambers (DESAGA).

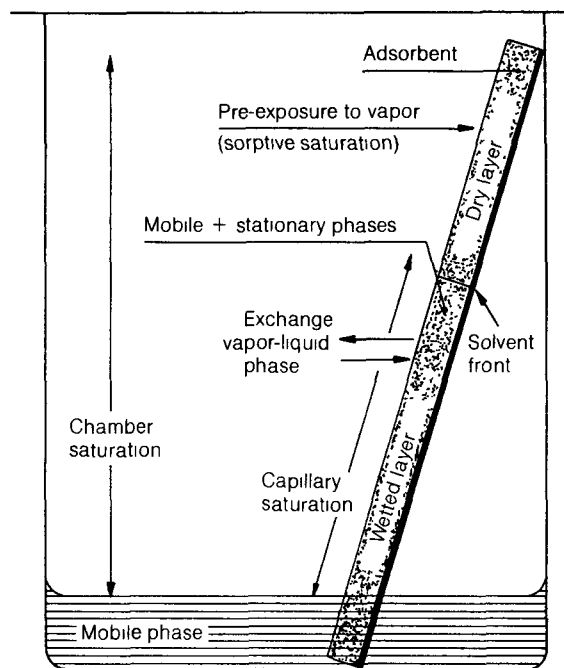


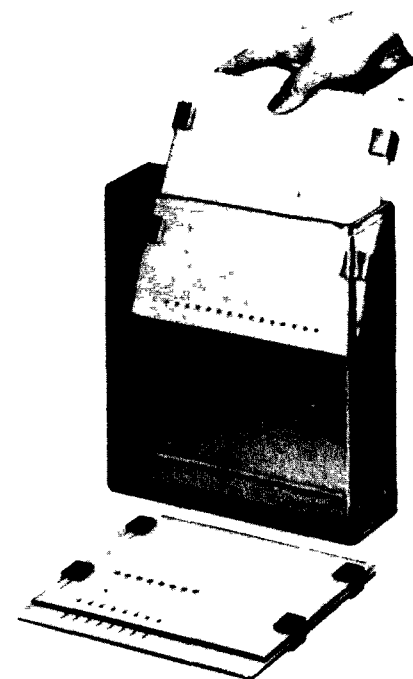
Fig. 57: Schematic representation of the relationships between development, chamber saturation and pre-loading with solvent vapors (acc. [8]).

But there can be no question of chamber saturation if the TLC plate is then placed directly in the chamber. But at least there is a reduction in the evaporation of mobile phase components from the layer. Mobile phase components are simultaneously transported onto the layer (Fig. 57). In the case of multicomponent mobile phases this reduces the formation of β -fronts.

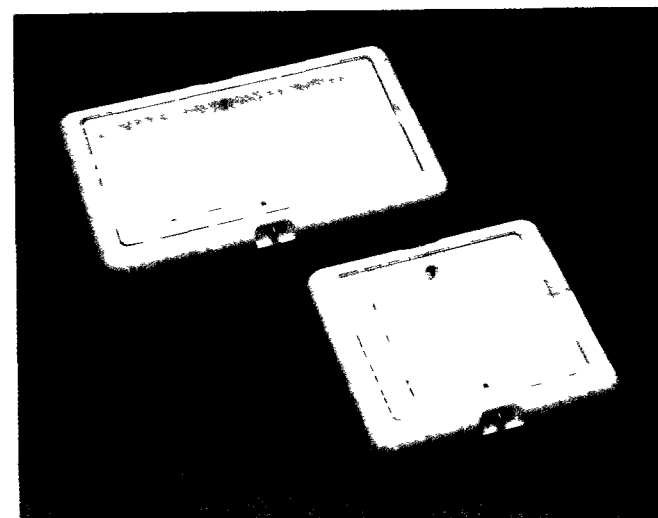
Apart from such trough chambers there are also S-chamber systems (small chambers, sandwich chambers) with deliberately reduced vapor volumes, which are specially suited to adsorptive separations. Such chambers are available for vertical and horizontal development (Fig. 58). Different separation results are naturally obtained in trough and S-chambers [8].

The description of the experiment must, therefore, state what type of chamber was used and whether "chamber saturation" was employed.

The twin-trough chamber (Fig. 43A) was not just developed to economize in mobile phase; it also allows the layer to be impregnated as desired from the vapor



A



B

Fig. 58: A) S chamber, B) horizontal developing chamber (CAMAG)

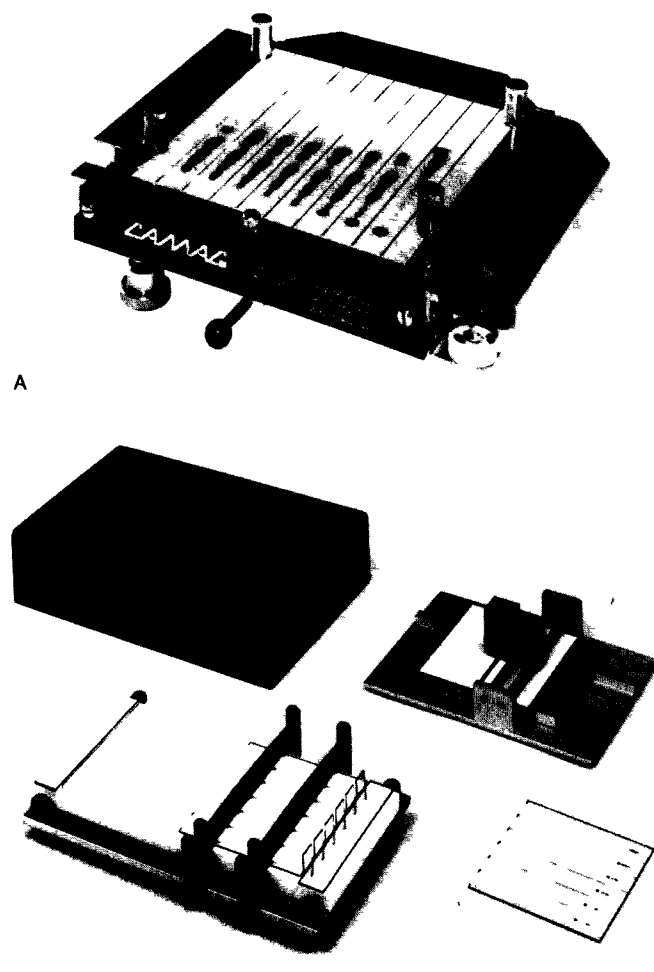


Fig. 59: Vario chambers (CAMAG) for plate format 20 cm × 20 cm (A) and 10 cm × 10 cm (B)

phase. In the case of acidic or basic mobile phases a demixing of the mobile phases usually occurs in the lower part of the chromatogram during development. When, however, a vapor such as acetic acid or ammonia has access to the stationary phase it is often possible to substitute an acid- or ammonia-containing mobile phase by a neutral one. The impregnating liquid is placed in one of the troughs, the mobile phase and the TLC/HPTLC in the other one.

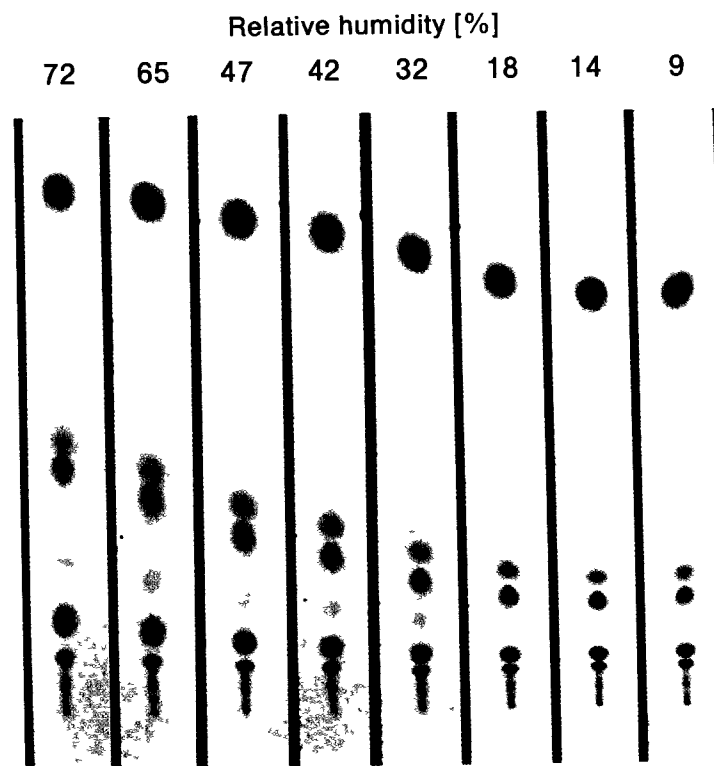


Fig. 60: Chromatogram of a 6 dyestuff mixture made up (according to falling R_f values) of Sudan red 7B, Sudan orange G, Sudan black B, Sudan yellow, Artisil blue 2RP and Sudan black B (2 components) under different humidity conditions. From left to right 72, 65, 47, 42, 32, 18, 14 and 9% relative humidity; layer: silica gel 60; mobile phase: toluene.

A precise mastery of the chromatographic process also requires that the relative humidity be controlled. There are sufficient examples demonstrating that reproducible development is only possible if temperature and relative humidity are maintained constant. The influence of the latter on chromatographic behavior can be investigated using the Vario KS chamber (Fig. 59). When the relative humidity is altered it is possible that not only the zone behavior will be changed but also the order of the zones on the chromatogram (Fig. 60).

It is possible to control the relative humidity with sulfuric acid solutions of particular concentrations (Tab. 26) or with saturated salt solutions in contact with excess salt (Tab. 27). These liquids are placed in a conditioning chamber

Table 26: Relative humidities over various concentrations of sulfuric acid at 20°C.

Sulfuric acid concentration [%]	Relative humidity [%]	Manufacture: ml conc. sulfuric acid + ml water
98	0	100 + 0
62.5	9	100 + 60
55	15	100 + 82
50	20	100 + 100
40	32	68 + 100
36	42	57 + 100
33	47	50 + 100
28	58	39.5 + 100
25	65	34 + 100
22	72	27.5 + 100

Table 27: Relative humidities over saturated salt solutions in contact with undissolved salts [8].

Salt	Rel. humidity [%]	Salt	Rel. humidity [%]
ZnCl ₂ · 1½ H ₂ O	10	NaNO ₂	66
LiCl · H ₂ O	15	CuCl ₂ · 2 H ₂ O	67
CaBr ₂	16.5	NH ₄ Cl + KNO ₃	72.6
K(HCOO)	21.3	NaClO ₃	75
K(CH ₃ COO)	22.7	NaCl	75.7
NiBr ₂	27.1	H ₂ C ₂ O ₄ · 2 H ₂ O	76
NaCl + KNO ₃ + NaNO ₃ (16°C)	30.5	Na(CH ₃ COO) · 3 H ₂ O	76
KF	30.5	Na ₂ S ₂ O ₃ · 5 H ₂ O	78
MgBr ₂	31.8	NH ₄ Cl	79.2
CaCl ₂ · 6 H ₂ O	32.3	(NH ₄) ₂ SO ₄	79.5
MgCl ₂ · 6 H ₂ O	32.4	KBr	84
NaCl + KNO ₃ (16°C)	32.6	KCl	85
CrO ₃	35	KHSO ₄	86
NaSCN	35.7	BaCl ₂ · 2 H ₂ O	88
NaCl + KClO ₃ (16°C)	36.6	K ₂ CrO ₄	88
NaI	38.4	ZnSO ₄ · 7 H ₂ O	90
Zn(NO ₃) ₂ · 6 H ₂ O	42	Na ₂ CO ₃ · 10 H ₂ O	90
K ₂ CO ₃ · 2 H ₂ O	44	NaBrO ₃	92
KNO ₂	45	K ₂ HPO ₄	92
KSCN	47	Na ₂ SO ₄ · 10 H ₂ O	93
NaHSO ₄ · H ₂ O	52	NH ₄ H ₂ PO ₄	93
Na ₂ Cr ₂ O ₇ · 2 H ₂ O	52	KNO ₃	93.2
Ca(NO ₃) ₂ · 4 H ₂ O	54	Na ₂ HPO ₄ · 12 H ₂ O	95
Mg(NO ₃) ₂ · 6 H ₂ O	54	Na ₂ SO ₃ · 7 H ₂ O	95
FeCl ₂ · 4 H ₂ O	55	K ₂ SO ₄	97.2
NaBr · 2 H ₂ O	58	CaSO ₄ · 5 H ₂ O	98
		Pb(NO ₃) ₂	98

(Fig. 43B) or in one of the troughs of a twin-trough chamber (Fig. 43A). If a conditioning chamber is employed the equilibrated TLC plate must then be transferred to the chromatography tank without delay. When a twin-trough chamber is employed the chromatography can be started after equilibration has taken place merely by adding the mobile phase to the second trough.

Such details must also be documented in order to make it possible for others to repeat the experiment.

4.2 Documentation on the Chromatogram

One of the great advantages offered by thin-layer chromatography is that several samples (the same or different) can be developed on the same TLC/HPTLC plate together with the appropriate reference substances. A horizontal chamber (Fig. 58B) can be used to generate up to 70 chromatograms simultaneously, with development taking place antiparallel from two opposing edges of the plate and requiring less than 20 ml of mobile phase.

Samples and reference substances should be dissolved in the same solvents to ensure that comparable substance distribution occurs in all the starting zones. In order to keep the size of the starting zones down to a minimum (diameter TLC: 2 to 4 mm, HPTLC: 0.5 to 1 mm) the application volumes are normally limited to a maximum of 5 µl for TLC and 500 nl for HPTLC when the samples are applied as spots. Particularly in the case of adsorption-chromatographic systems layers with concentrating zones offer another possibility of producing small starting zones. Here the applied zones are compressed to narrow bands at the solvent front before the mobile phase reaches the active chromatographic layer.

The use of application schemes and labelling each single chromatogram track can avoid mistakes as far as the order of application and positioning are concerned. Each sample and reference solution is applied twice with a displacement of half a plate width in the data-pair method [9], that has been in use since the 1960s for the purpose of reducing errors of application and chromatography (Fig. 61). The position of application must naturally be chosen so that the starting zone does not dip into the mobile phase.

A soft pencil can be used to write on the chromatogram. But the chromatogram should not be marked *below* the starting point, because the layer could be damaged

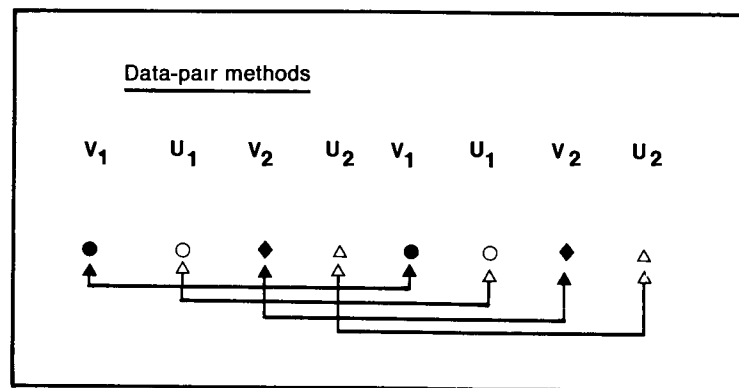


Fig. 61: Application scheme according to the data pair method — V_1 to V_3 = standard solutions, U_1 to U_3 = sample solutions

with the result that the substance distribution in the chromatographic zones could be affected. This would lead to errors in direct photometric analysis [10]. It is best to label the chromatogram above the level of the solvent front when the chromatogram is later developed. Immediately after development is completed the level of the solvent front should be marked at the left and right-hand edges of the plate to allow calculation of the R_f values. This should be done even if a solvent front “monitor” is employed [11] or the development is controlled by means of an AMD [12–15] or PMD system [16–18]. The practice of cutting a scratch across the whole adsorbent layer for this purpose is now obsolete — especially doing it before development.

The type of plate, chamber system, composition of mobile phase, running time and detection reagent used must naturally all be recorded. The sample protocol illustrated in Figure 62 can be employed.

When preparing mobile phase mixtures each individual component should be measured out separately and only then placed in the mixing vessel. This prevents not only contamination of the solvent stock by vapors from the already partially filled mixing vessel (e.g. ammonia) but also volumetric errors caused by volume expansions or contractions on mixing.

All details in the reagent monographs concerning mobile phases are given in parts by volume unless it is specifically stated otherwise.

Test	Test No
	Name
	Date
Chromatographic conditions:	
Method	
Stationary phase	
Mobile phase	
Migration distance	Migration time
Detection	a)
	b)
	c)
Evaluation and comments	
Preparation of sample Volume applied Preparation of standards Volume applied	

Fig. 62: Example of a protocol form

4.3 Fixing the Visual Appearance of the Chromatogram

A chromatogram is produced by developing a TLC/HPTLC plate, but it may be necessary to employ one of the reagents described to make the positions, structures and sizes of the chromatogram zones apparent so that they can be recorded. If the R_f values are the same a comparison of the sizes of the zones of the sample and standard substances gives an indication for estimating the amounts. If, as a result of matrix effects, the R_f values of sample and standard are not the same then their

zone size and distribution of substance on the chromatogram will also differ on account of different diffusion patterns. These facts also belong in the protocol of a chromatographic separation.

It is usually only possible to store the original chromatogram if TLC foils are employed or if the adsorbent layer is fixed and removed from the plate as a whole. This can be achieved by treating the chromatogram with collodium [19], “adhesive” [20] or plastic dispersions and exercising a little patience.

4.3.1 Preserving with Neatan

Chromatograms can be made “handleable” and storable by treatment with plastic dispersions, based on polyacrylic ester, polyvinyl chloride or polyvinyl propionate, such as, for example, Neatan (MERCK) [21–23]. In order to avoid clogging the spray head with plastic dispersion residue it is recommended that it be rinsed through immediately after use with tetrahydrofuran or that disposable jets be employed [24].

After drying at a temperature below 50 °C (higher temperatures lead to yellowing!) an adhesive film is rolled over the layer and the covered chromatogram cut down to the glass plate with a razor blade within 2 to 3 mm of its edges and raised at one corner. The “polymerized” layer can then be detached from the glass plate by soaking in water (methanol in the case of polyamide layers [23]) and after drying stuck into the laboratory notebook and labelled (take care when aggressive reagents have been used!).

This method of chromatogram preservation has lost a great deal of its importance with the increasing perfection of photographic methods, particularly since true-colored, instant, paper and slide positives have become available. Photography is the more rapid method of documentation if suitable photographic equipment is available.

4.3.2 Documentation by Sketching, Photocopying or Photographing

The phenomenological analysis results can be recorded by sketching or tracing on translucent paper and colored with crayons or pens to reproduce the impression of color. It is usual to mark fluorescent zones with a “ray-like” edge; phosphorescence-inhibiting zones are marked with a broken line. Stippling and hatching etc. can also be employed by a working group to convey additional information.

Direct copying on Ozalid or Ultrarapid blue print paper has also been employed for documentation [25–27]. For this purpose the chromatograms are laid layer-side up on a light box and, if necessary, covered with a sheet of 1 mm thick glass. The Ozalid paper is then laid on with the yellow-coated side down and covered with an appropriately shaped piece of wood (to keep out the daylight and to maintain contact). The exposure time is 8 to 10 minutes depending on the chromatographic background; it can be shortened to 3 to 4 minutes for weakly colored zones or lengthened appropriately for heavily colored backgrounds. The Ozalid paper is exposed to ammonia vapor after exposure. The chromatogram zones are recorded as red-violet spots on a pale background. AGFA-Copyrapid CpP or CpN papers can also be employed [37].

The simplest method of recording chromatograms nowadays is by means of conventional photocopying (Fig. 63).

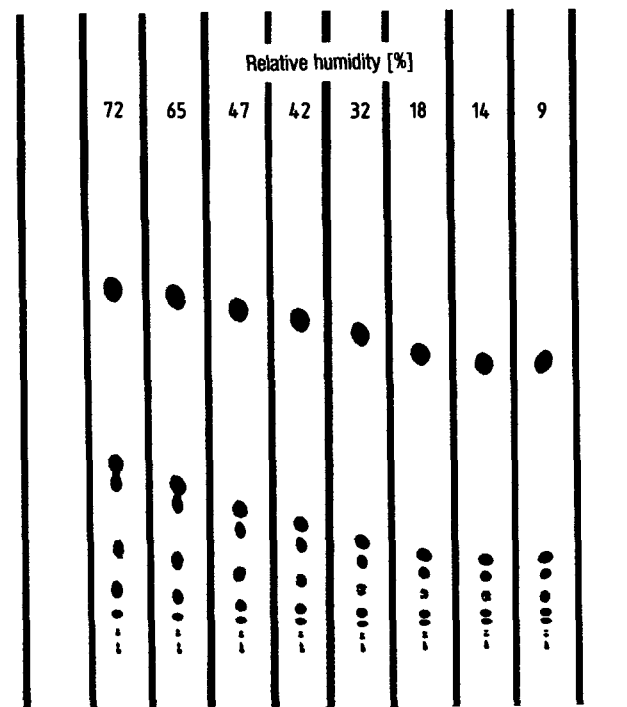


Fig. 63: Photocopy of the chromatogram in Fig. 60.

The chromatograms can also be recorded onto normal photographic paper (e.g. Ilfospeed 2.1 M, glossy 2, Ilford, Essex [28] or Kodak paper [29]) by transmitted light. Here too, as in the case of copying onto Ozalid paper, a sandwich is prepared from chromatogram and photographic paper (dark room!); this is then illuminated for about 1 s with a 25 watt incandescent bulb at a distance of 1.5 m. The negative image on the paper is then developed.

Color reproduction of the chromatograms can be achieved by color photography – the best, but also the most expensive method of documenting thin-layer chromatograms. It can be used not only to produce true-color reproductions of colored zones but also – with the aid of a Reprostar (Fig. 64) or a UVIS analysis lamp (Fig. 6) – of fluorescent or fluorescence-quenched zones. When photograph-

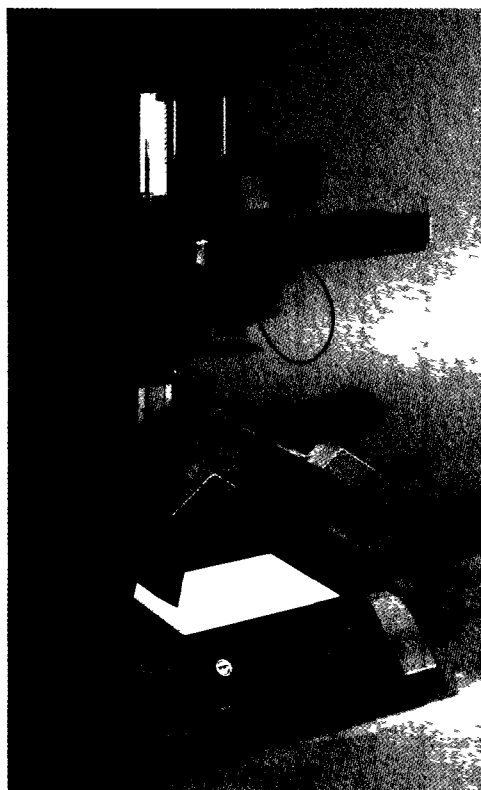


Fig. 64: Reprostar (CAMAG).

ing fluorescent zones it is necessary to ensure that the filter transparency of the UV lamp is optimal so that the chromatogram illumination is even (Sec. 2.2.2 and Fig. 8). It must also be ensured that no exciting radiation reaches the film [31 – 33].

When fluorescence-quenched zones are being photographed (excitation at $\lambda = 254$ nm) it is often sufficient to place a Type GG 435 glass filter (SCHOTT, thickness 4 mm) in front of the camera lens. On the other hand, a Wratten Type 2 E filter is recommended for recording fluorescent zones (excitation at $\lambda = 365$ nm) [34]. The slight blue coloration of the film caused by light passing the black-light filter of the UV lamp can be avoided by a yellow or pale orange filter.

The required exposure times are difficult to estimate. They are best found by trial and error. Documentation of fluorescence quenching at $\lambda = 254$ nm usually only requires one trial. The exposure time found to be adequate here is normally suitable for all following exposures of fluorescence quenching if the exposure conditions are maintained constant (camera type, film type, distance of objective and lamp, aperture etc.). The exposure time required for fluorescent chromatograms is primarily dependent on the intensity of the fluorescence and, therefore, has to be optimized for each chromatogram. It is best to operate with a range of exposure times, e.g. aperture 8 with exposures of 15, 30, 60, 120 and 240 seconds. Experience has shown that one exposure is always optimal.

Favorable settings are given in Table 28 for the employment of Polaroid color film Type 669 in a CU-5 Polaroid-Land camera with a 127 mm objective. Further

Table 28: Setting conditions for a Polaroid Land CU 5 camera with supplementary lens (0.5 dioptries) for photographing TLC plates of 20 × 20 cm format on Type 669 Polaroid color film.

Subject	Aperture	Exposure	Color correction
Colored zones, white transmitted <i>or</i> incident light	11	1/8 s	no
Colored zones, white transmitted <i>and</i> incident light	16	1/8 s	no
Fluorescent zones, incident light at $\lambda = 365$ nm	8	30 s	yes
Fluorescence quenching zones, incident light at $\lambda = 254$ nm	8	30 s	yes

detailed data have been reported in the literature for other types of cameras and films [30–32, 35, 36]. The production of autoradiograms will be dealt with in Volume 2.

The color plates for the chromatograms reproduced in the reagent monographs were produced using an Olympus OM-4 camera with 50 mm lens combined with a copying stand with TTL-Makroblitz T 28 (OLYMPUS).

4.4 Documentation by Means of in situ Evaluation by Computer

The methods of documentation of thin-layer chromatograms described until now depend on the photographic recording of the visually detectable chromatograms. The film negatives or slides can then be subjected to densitometric quantitation [26, 38, 39] and included in the protocol. This indirect method of TLC chromatogram quantitation with its intermediate photographic step has been rendered completely obsolete with the coming of the computer-controlled, chromatogram spectrometer.

Particularly elegant documentation is achieved by storing the quantitative TLC data on diskettes. They are then available for years and complement the qualitative record in an excellent manner. In addition they are always available for statistical analysis and, thus, contribute to comprehensive documentation.

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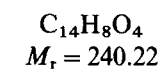
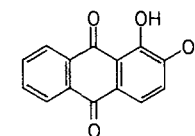
Part II

Reagents in Alphabetical Order

Alizarin Reagent

Reagent for:

- Cations [1 – 7]



Preparation of the Reagent

Dipping solution Dissolve 100 mg alizarin in 100 ml ethanol.

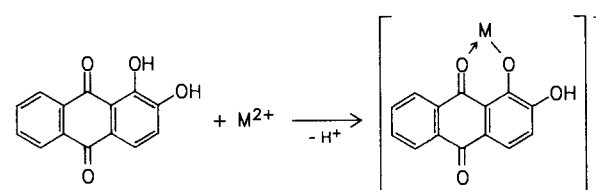
Spray solution A 0.25% [3] or saturated solution of alizarin [4] in ethanol.

Storage The solution may be kept for several days at room temperature.

Substances Alizarin
Ethanol
Ammonia solution (25%)

Reaction

Metal cations yield colored complexes with alizarin:



Method

The chromatogram is dried for 10 min in warm air and either immersed in the dipping solution (1 s) or sprayed evenly with the spray solution. The still-moist TLC plates are placed in the empty part of a twin-trough chamber filled with ammonia solution (25%) for 1 min. In a few minutes red-violet zones appear on a violet background. If the plate is then dipped in either 0.1–1% acetic acid in diethyl ether or in a 1% solution of boric acid in methanol-water (9 + 1) the background turns yellow and most of the chromatogram zones appear as red to violet spots. The following cations (arranged as in the periodic table) can be detected:

I	II	III	IV	V	VI	VII	VIII
Li	Be			NH ₄			
	Mg	Al					
Cu	Ca Zn	Ga Sc	Ti	As V	Se Cr	Mn	Fe Co Ni
Ag	Sr Cd	In	Sn Zr	Sb			Pd
Au	Ba Hg	La	Pb	Bi			Pt

and Ce, Th, U and other rare earths.

Note: The reagent can be employed on silica gel layers, which may also be impregnated, for example, with 8-hydroxyquinoline or dibenzoylmethane [3] or with 2,2'-dipyridyl or iminodiacetic acid [4] or on cellulose layers.

Rather than dipping the chromatogram in acid solution it is preferable to heat it to 100°C for 2–5 min (fume cupboard!) in order to evaporate the ammonia and turn the background yellow. By this means it is possible to increase the sensitivity of detection for some of cations e.g. Sr^{2+} and Ba^{2+} . However, these zones fade after some time, so that it is necessary to quantify the chromatogram immediately after heating.

Procedure Tested

Nickel, Copper and Beryllium nitrates; Calcium, Magnesium, Strontium and Barium chlorides [5, 7]

Method	One-dimensional ascending development in a HPTLC chamber with chamber saturation.
Layer	HPTLC plates Cellulose (MERCK). The plates were pre-washed with mobile phase and dried for 10 min in a stream of warm air before use.
Mobile phase	1. Ni, Cu and Be cations: acetone – nitric acid (25%) (35 + 15). 2. Alkaline earth cations [5]: methanol-hydrochloric acid (25%) (80 + 20).
Migration distance	5–7 cm
Running time	20 min

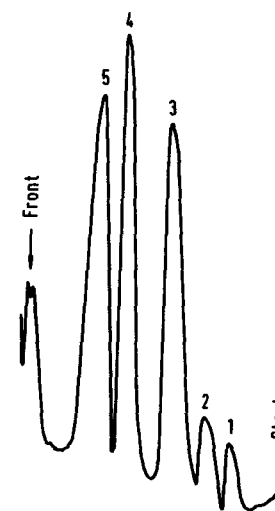


Fig. 1: Absorption scanning curve of the alizarin complexes of barium (1), strontium (2), calcium (3), magnesium (4) and beryllium cations (5). The amounts applied were 2 µg in each case.

Detection and result: The separation was adequate in the systems given. The following hR_f values were obtained after heating for 2–5 min to 100°C.

Mobile phase 1: Ni^{2+} (blue-violet), hR_f : 45–50; Cu^{2+} (violet), hR_f : 55–60; Be^{2+} (violet), hR_f : 80–85.

Mobile phase 2: Ba^{2+} (pale blue-violet), hR_f : 15–20; Sr^{2+} (pale blue-violet, slightly tailing), hR_f : 30–35; Ca^{2+} (violet), hR_f : 50–55; Mg^{2+} (violet), hR_f : 75–80; Be^{2+} (violet), hR_f : 93–98.

The visual limit of detection was between 30 and 50 ng per chromatogram zone for nickel and copper, a factor of ten worse for the alkaline earths. There was sometimes an “impurity” front in the same hR_f range as beryllium.

In situ quantitation: The photometric determination was made in reflectance mode at $\lambda = 550$ nm (Fig. 1).

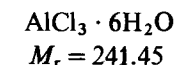
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Aluminium Chloride Reagent

Reagent for:

- Unsaturated 1,2- and 1,3-dihydroxyketones, e.g. flavonoids [1–4]
- Mycotoxins: zearalenone [5, 6, 10]; ochratoxin [5]; sterigmatocystine [7–11]; citrinine [12]
- Trichothecenes: deoxynivalenol (vomitoxin) [13]
- Cholesterol and its esters [14]
- Phospholipids and triglycerides [14]

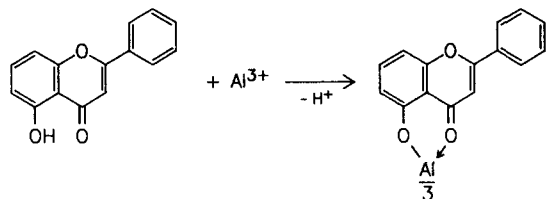


Preparation of the Reagent

Solution I	Dissolve 0.2 to 1 g aluminium chloride in 100 ml ethanol.
Solution II	Dissolve 20 g aluminium chloride in 100 ml ethanol.
Storage	The solutions can be stored for long periods in the refrigerator.
Substances	Aluminium chloride hexahydrate Ethanol absolute

Reaction

Aluminium chloride forms, for example, fluorescent complexes with flavonoids:



Method

The developed chromatograms are briefly immersed in or evenly sprayed with the appropriate reagent solution. Solution I is employed for flavonoids [1, 3] and solution II for mycotoxins [5, 8, 12], phospholipids, triglycerides and cholesterol [14].

After the dipped or sprayed chromatogram has been dried in a stream of cold air long-wave UV light ($\lambda = 365 \text{ nm}$) reveals fluorescent yellow zones (flavonoids). Sterigmatocystine, which can be detected without derivatization on account of its red intrinsic fluorescence (detection limit $0.5 \mu\text{g}$), also fluoresces pale yellow after being heated to 80°C [9] or 100°C [13] for 10 min; on the other hand, citrinine, zearalenone and vomitoxin fluoresce blue.

The detection limits are ca. 20 ng per chromatogram zone.

Note: The reagent can be employed on silica gel, kieselguhr, polyamide, RP, CN, NH_2 and cellulose layers.

The colors of the fluorescing zones can depend on the concentration of the aluminium chloride solution.

Hypericin which is a hydroxyanthraquinone and the antibiotic nystatin also yield fluorescent zones. A higher fluorescence intensity is frequently obtained by heating to 88°C for 2–5 min instead of simply allowing to dry at room temperature.

Procedure Tested

Flavonoids (Quercetin, Rutin, Hyperoside, Quercitrin) [15]

Method	One-dimensional, ascending development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK) pre-washed by a single development with chloroform-methanol (50+50). The layers were preconditioned for 30 min over water after the sample had been applied.
Mobile phase	Ethyl acetate – formic acid (98–100%) – water (85 + 10 + 15).
Migration distance	6 cm
Running time	18 min

Detection and result: The chromatogram was freed from mobile phase and dipped for 1 s in solution I and after drying for 1 min in a stream of cold air it was dipped in a solution of liquid paraffin – *n*-hexane (1 + 2) in order to stabilize and increase the intensity of fluorescence by a factor of 1.5–2.5. The derivatives which were pale yellow in daylight after drying fluoresce pale blue to turquoise in long-wave

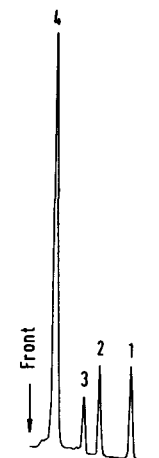


Fig. 1: Separation of flavonoids: fluorescence scanning curve of rutin (1), hyperoside (2), quercitrin (3) and quercetin (4).

UV light ($\lambda = 365$ nm): rutin (hR_f : 25–30), hyperoside (hR_f : 45–50), quercitrin (hR_f : 60–65) and quercetin (hR_f : 85–90). The detection limits were 10 ng substance per chromatogram zone.

In situ quantitation: The in situ fluorescence measurement was carried out at $\lambda_{exc} = 436$ nm and $\lambda_{em} = 546$ nm (monochromate filter M 546).

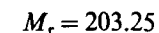
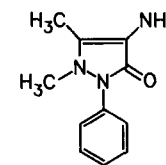
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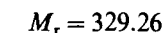
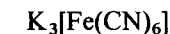
4-Aminoantipyrine – Potassium Hexacyanoferrate(III) Reagent (Emerson Reagent)

Reagent for:

- Arylamines [1, 2]
- Phenols [3–5]
- Salithion [6]
- Sympathicomimetics



4-Aminoantipyrine



Preparation of the Reagent

Dipping solution I Dissolve 1 g 4-aminoantipyrine (4-aminophenazone; 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) in 100 ml 80% ethanol.

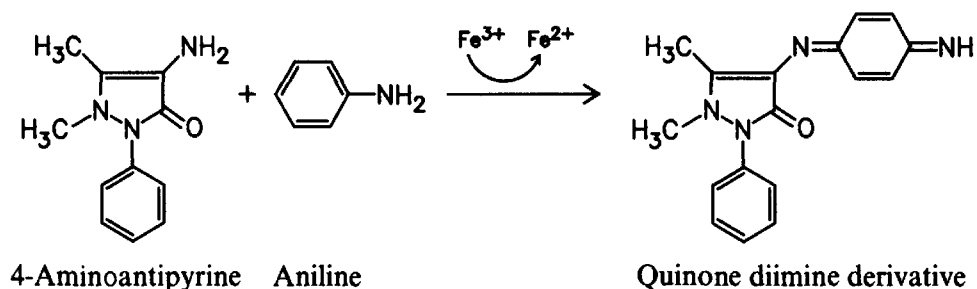
Dipping solution II Dissolve 4 g potassium hexacyanoferrate(III) in 50 ml water and make up to 100 ml with ethanol.

Spray solution I Dissolve 2 g 4-aminoantipyrine in 100 ml 80% ethanol.

Spray solution II See dipping solution II.

Storage The dipping solution and spray solution I can be stored in the refrigerator for about 1 week.

4-Aminoantipyrine forms with aniline, for instance, a colored diimine derivative under the oxidative influence of iron(III) ions.



Method

The chromatogram is freed from mobile phase and immersed in dipping solution I for 1 s or sprayed with spray solution I, dried in warm air for 5 min and then immersed for 1 s in dipping solution II or sprayed with spray solution II. After redrying the background is decolorized by placing the chromatogram in a twin-trough chamber, one of whose troughs contains 25% ammonia solution. Red-orange colored zones are produced on a pale yellow background. The color intensity of the chromatogram zones is also increased in the case of phenols, since these only react in alkaline medium [4].

Note: The reagent can be just as successfully employed on silica gel, kieselguhr, aluminium oxide and polyamide layers as it can with RP and NH₂ phases. The final treatment with ammonia vapor to decolorize the background can be omitted in the last case.

An iodine solution can be employed as oxidizing agent in place of potassium hexacyanoferrate(III). 4-Aminoantipyrine also produces colored zones with 1- and 1,4-unsaturated 3-ketosteroids (pregnadienediol derivatives) in the absence of oxidizing agents.

Method	Ascending, one chamber with cl (5 min intermed required for the
Layer	TLC plates Silic WRF _{254s} (MERC NAGEL).
Mobile phase	TLC: chloroform (separation).
Migration distance	TLC: 10 cm; HI
Running time	TLC: 20 min; H

Detection and result: The following *hl* carvacrol and thymol (red) 30–35, eu ground was pale yellow colored. The chromatogram zone. The colored zone

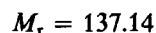
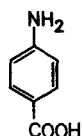
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4-Aminobenzoic Acid Reagent

Reagent for:

- Carbohydrates (sugars)
- e.g. monosaccharides [1 – 5]
- disaccharides [2, 3]
- uronic acids [1 – 3]



Preparation of the Reagent

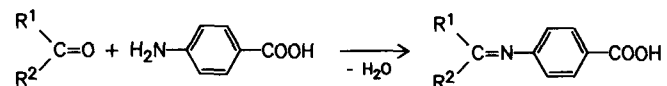
Dipping solution Dissolve 1 g 4-aminobenzoic acid in 18 ml glacial acetic acid and add 20 ml water and 1 ml 85% phosphoric acid; immediately before use dilute with acetone in the ratio 2 + 3 [2].

Storage The reagent may be stored for 1 week in the dark at room temperature.

Substances 4-Aminobenzoic acid
Glacial acetic acid
Orthophosphoric acid (85%)
Acetone

Reaction

Sugars react with the reagent probably with the formation of SCHIFF's bases:



Method

The chromatogram is freed from mobile phase and dipped in the reagent for 2 s or uniformly sprayed with it, dried for several minutes in a stream of cold air and heated to 100°C for 10 – 15 min. The result is reddish-brown chromatogram zones on a colorless to pale brown background.

Note: The reagent can be employed on cellulose layers. Sodium acetate-buffered kieselguhr layers are less suitable [6]. Only a few sugars are detectable and those with lower sensitivity if acid is not added to the reagent [7].

Procedure Tested

Sugar in Diabetic Chocolate [8]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 (MERCK).
Mobile phase	Dichloromethane – methanol – glacial acetic acid – water (50 + 50 + 25 + 10).
Migration distance	15 cm
Running time	120 min

Detection and result: The chromatogram was freed from mobile phase and immersed in the reagent solution for 2 s and placed in a drying cupboard while still moist. After heating to 120°C for 15 min red-brown (fructose) and grey-blue (lactose) chromatogram zones were produced, which fluoresced turquoise under long-wavelength UV light ($\lambda = 365$ nm). The detection limits in visible light were 200 – 300 ng substance per chromatogram zone. The detection limits were appreciably lower with less than 5 ng per chromatogram zone on fluorimetric analysis.

In situ quantitation: Quantitation was performed fluorimetrically ($\lambda_{\text{exc}} = 365$ nm, $\lambda_{\text{fl}} > 560$ nm). The baseline structure was most favorable under the chosen conditions (Fig. 1).

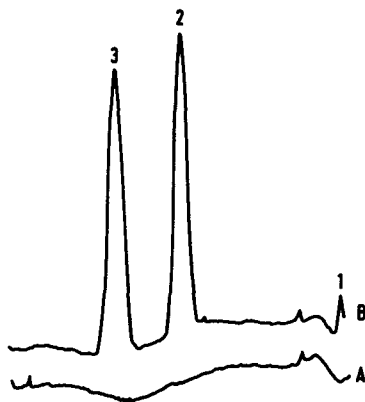


Fig. 1: Fluorescence plot of a blank (A) and a chromatogram track of a diabetic chocolate extract (B). Circa 50 ng lactose and fructose were applied. Start (1), lactose (2), fructose (3).

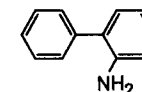
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2-Aminodiphenyl – Sulfuric Acid Reagent

Reagent for:

- Carbonyl compounds [1, 2]
e.g. aliphatic aldehydes from C-8,
glycol aldehyde [5], glyoxalic acid,
2,3-pentanedione



- Vitamin B₆ (pyridoxal) [2]

$C_{12}H_{11}N$

H_2SO_4

- Sugars [3, 4]

$M_r = 169.23$

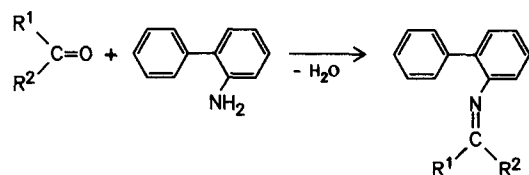
$M_r = 98.08$

Preparation of Reagent

- Solution I** Dissolve 1 g 2-aminodiphenyl (biphenyl-2-ylamine) in 100 ml ethanol.
- Solution II** 20% sulfuric acid.
- Dipping solution** Carefully mix equal volumes of solutions I and II immediately before the dipping process.
- Spray solution** For sugars [3]: dissolve 3 g 2-aminodiphenyl in 100 ml glacial acetic acid and add 1.5 ml 85% orthophosphoric acid.
- Storage** Solution I may be stored in the refrigerator for several days.
- Substances** Biphenyl-2-ylamine
Sulfuric acid (25%)
Ethanol
Glacial acetic acid
Orthophosphoric acid (85%)

Reaction

2-Aminodiphenyl reacts with carbonyl compounds to form colored or fluorescent SCHIFF's bases with the elimination of water:



Method

The chromatogram is freed from mobile phase and immersed for 1 s in the freshly prepared reagent solution and then heated to 105 to 110°C for 5 to 10 min. Green, blue or purple fluorescence appears on a dark background under long-wavelength UV light ($\lambda = 365$ nm).

Note: The dipping solution, which can also be used as a spray solution, can be employed with silica gel, kieselguhr, cellulose, RP, NH₂ and CN phases. Sugars (exceptions include, for example, fructose, melezitose and raffinose) yield brilliantly colored zones on an almost colorless background when the spray solution is employed. Aldohexoses appear brown, aldopentoses bright red and hexuronic acids orange in color [3]. The detection limit differs for different substances; it ranges from 10 ng (pyridoxal) over 100 ng (cinnamaldehyde) up to 2 µg (citral).

Procedure Tested

Citral, Citronellal, Cinnamaldehyde [6]

Method	Ascending, one-dimensional development in a trough chamber.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene — ethyl acetate — glacial acetic acid (90 + 5 + 5).

Migration distance 5 cm

Running time 20 min

Detection and result: The chromatogram was freed from mobile phase and immersed for 1 s in the freshly prepared dipping solution and then heated to 105 to 110°C for 5 to 10 min. Citral (hR_f 60) and citronellal (hR_f 80) produced brown zones on a light brown background in visible light, the zones had a purple fluorescence under long-wavelength UV light ($\lambda = 365$ nm). Cinnamaldehyde acquired an intense yellow color but did not fluoresce.

In situ quantitation: This could be made under long-wavelength UV light ($\lambda_{exc} = 365$ nm; $\lambda_{fl} > 560$ nm). However, it was not very sensitive.

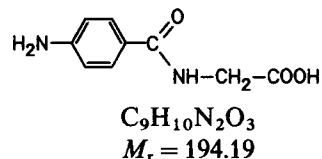
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4-Aminohippuric Acid Reagent

Reagent for:

- Sugars (monosaccharides) [1, 2]

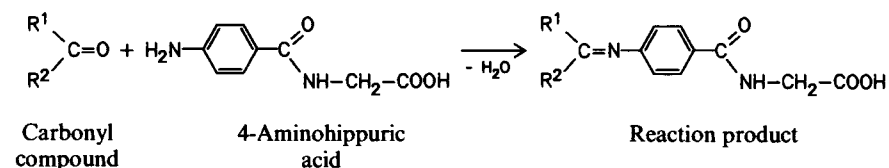


Preparation of the Reagent

Dipping solution	Dissolve 0.5 g 4-aminohippuric acid in 50 ml ethanol and make up to 100 ml with toluene.
Spray solution	Dissolve 0.5 g 4-aminohippuric acid in 100 ml ethanol.
Storage	Both solutions are stable for several days.
Substances	4-Aminohippuric acid Ethanol Toluene

Reaction

Sugars react with the reagent, probably with the production of SCHIFF's bases:



Method

The chromatogram is freed from mobile phase and immersed in the reagent solution for 1 s or homogeneously sprayed with the spray solution and then heated to 140°C for 8 min [2]. Hexoses and pentoses yield orange-colored zones on an almost colorless background, the zones fluoresce blue under long-wavelength UV light ($\lambda = 365$ nm).

Note: The layers on which the reagent can be employed include silica gel, cellulose and polyamide.

Procedure Tested

Hexoses and Pentoses [1]

Method	Ascending, one-dimensional development in a HPTLC trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	2-Propanol – 0.75% aqueous boric acid – glacial acetic acid (40 + 5 + 1).
Migration distance	8 cm
Running time	80 min

Detection and result: The chromatogram was freed from mobile phase and immersed in the reagent solution for 1 s and heated to 140°C for 10 min. The following appeared as blue fluorescent zones under long-wavelength UV light ($\lambda = 365$ nm): lactose (hR_f 25–30), fructose (hR_f 30–35), arabinose (hR_f 45–50), xylose (hR_f 55–60) and rhamnose (hR_f 60–65) (Fig. 1A).

Immersion in a liquid paraffin – *n*-hexane (1 + 3) did not lead to an appreciable increase in fluorescence intensity.

In situ quantitation: The fluorimetric analysis was made in UV light ($\lambda_{exc} = 313$ nm, $\lambda_{fl} > 460$ nm; F1 46 filter). The signal-noise ratio was better above $\lambda = 460$ nm than when a F1 39 filter is employed.

The detection limits per chromatogram zone were 10–20 ng for xylose, 50–100 ng for fructose, arabinose and rhamnose and 1–2 μ g for lactose (Fig. 1B).

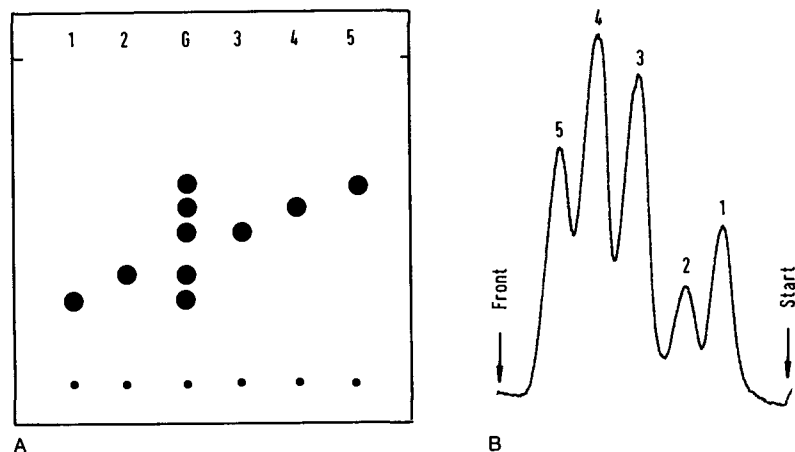


Fig. 1: Schematic diagram of the chromatographic separation (A) and the fluorescence scan (B) of a sugar mixture containing 1 µg substance per chromatogram zone. Lactose (1), fructose (2), arabinose (3), xylose (4), rhamnose (5), mixture (G).

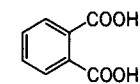
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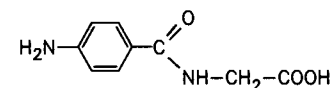
4-Aminohippuric Acid – Phthalic Acid Reagent

Reagent for:

- Sugars (mono- and disaccharides) [1 – 3]
 e.g. hydrolysates of tragacanth [4]



$C_8H_6O_4$
 $M_r = 166.13$
 Phthalic acid



$C_9H_{10}N_2O_3$
 $M_r = 194.19$
 4-Aminohippuric acid

Preparation of the Reagent

Dipping solution Dissolve 0.2 g 4-aminohippuric acid and 3 g phthalic acid in 50 ml ethanol and dilute with 50 ml toluene.

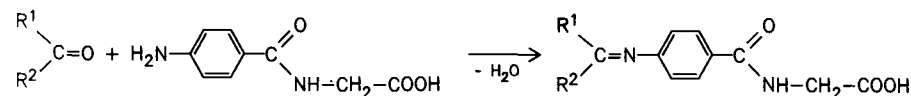
Spray solution Dissolve 0.3 g 4-aminohippuric acid and 3 g phthalic acid in 100 ml ethanol.

Storage Both solutions are stable for several days.

Substances
 4-Aminohippuric acid
 Phthalic acid
 Ethanol
 Toluene

Reaction

Sugars react with the reagent probably with the formation of SCHIFF's bases:



Method

The chromatogram is dried in a stream of warm air and immersed for 10 s in the reagent solution or the spray solution is applied to it homogeneously and it is then heated to 115–140 °C for 8–15 min [2, 4]. Yellow to orange-red zones are produced on an almost colorless background; these emit an intense blue fluorescence under long-wavelength UV light ($\lambda = 365$ nm).

Note: Subsequent immersion of the chromatogram in a mixture of liquid paraffin – *n*-hexane (1 + 2) leads to an increase in the fluorescence by a factor of 2.5 to 4.5 for some carbohydrates.

The visual limit of detection, when irradiating with UV light, is 250 ng per chromatogram zone; the zones are only detectable in visible light when the amounts are 4 to 5 times greater [2].

The reagent can be employed on silica gel, kieselguhr, Si 50 000, NH₂, cellulose and polyamide layers.

Procedure Tested

Glucose, Fructose, Maltose [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates NH ₂ F _{254s} (MERCK).
Mobile phase	Acetonitrile – water – phosphate buffer (pH = 5.9) (80 + 15 + 10). <i>Preparation of the phosphate buffer:</i> Dissolve 680 mg potassium dihydrogen phosphate in 50 ml water and add 4.6 ml 0.1 M caustic soda; then dilute 1 + 9 with water.
Migration distance	7 cm
Running time	20 min

Detection and result: The chromatogram was freed from the mobile phase, immersed in the reagent solution for 10 s and heated to 150 °C for 8 min. Maltose (*hR_f* 10–15), glucose (*hR_f* 20–25) and fructose (*hR_f* 25–30) appeared under

long-wavelength UV light ($\lambda = 365$ nm) as pale blue fluorescent zones on a weakly bluish fluorescent background.

The fluorescence intensity of the chromatogram zones could be stabilized and increased by a factor of 2.5 to 3.5 by subsequent immersion in liquid paraffin – *n*-hexane (1 + 2).

In situ quantitation: The in situ fluorimetric analysis was made under long-wavelength UV light ($\lambda_{\text{exc}} = 365$ nm, $\lambda_{\text{fl}} > 560$ nm) and is illustrated in Figure 1. The detection limits for maltose, glucose and fructose were ca. 10 ng substance per chromatogram zone.

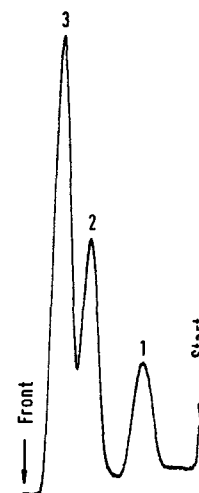


Fig. 1: Fluorescence plot for a sugar mixture containing 50 ng substance per chromatogram zone. Maltose (1), glucose (2), fructose (3).

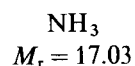
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Ammonia Vapor Reagent

Reagent for:

- Alkaloids
e.g. morphine, heroin [1, 2]
6-monoacetylmorphine [2]
- Mycotoxins
e.g. ochratoxin A [3–5]
- Flavonoids, flavonoid glycosides [6–9]
- Sennosides [10]
- Naphthoquinone glucosides [11]
- Valepotriates [12]
- Antibiotics
e.g. penicillic acid [13]
rifamycin [14]
tetracyclins [15]
- Anthracene derivatives [16]
- Homogentisic acid [17]



Preparation of Reagent

Solution	Ammonia solution (25%).
Storage	The reagent may be stored over an extended period.
Substances	Ammonia solution (25%)

Reaction

Morphine and heroin form fluorescent oxidation products on heating in the presence of ammonia [1].

Method

The chromatograms are dried in a stream of cold air (alkaloids: 110–120°C for 25 min in drying cupboard) and placed for 15 min in a twin-trough chamber — in the case of alkaloids while still hot — whose second trough contains 10 ml 25% ammonia solution.

Valepotriates are detected by placing the chromatogram 0.3 mm from a TLC plate sprayed with conc. ammonia solution (sandwich configuration layer to layer), fastening with clips and heating to 110°C in a drying cupboard for 10 min [12].

The result is usually chromatogram zones that fluoresce yellow, green or blue on a dark background under long-wavelength UV light ($\lambda = 365 \text{ nm}$), in some cases colored zones are detectable in visible light (e.g. homogentisic acid [17], sennosides [10], rifamycin [14]).

Note: The natural fluorescence colors of some flavonoids [7, 9] and anthracene derivatives [16] are altered by the ammonia treatment. This makes possible differentiation on the basis of color. Detection limits per chromatogram zone have been reported of 2 ng for morphine and heroin [2], 6 ng for ochratoxin A [5] and 1 μg for penicillic acid [13].

The reagent can be employed on silica gel, kieselguhr, Si 50 000, polyamide, RP and cellulose layers.

Procedure Tested

Alkaloids [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
---------------	----------------------------------------------------------------------------------------

Layer	HPTLC plates Silica gel 60 F _{254s} with concentrating zone (MERCK), which had been prewashed by developing once with chloroform – methanol (50 + 50) and then dried at 110°C for 30 min.
Application	The samples were applied to the concentrating zone as bands in the direction of chromatography. The zones were concentrated by brief development in the mobile phase described below almost to the junction between the concentrating zone and the chromatographic layer, followed by drying for 5 min in a stream of warm air. The actual chromatographic separation was then carried out.
Mobile phase	Methanol – chloroform – water (12 + 8 + 2).
Migration distance	4.5 cm
Running time	20 min

Detection and result: The chromatogram was heated in the drying cupboard to 110–120°C for 25 min and immediately placed – while still hot – in a twin-trough chamber, whose second trough contained 10 ml 25% ammonia solution, for 15 min. The chromatogram was then immersed for 2 s in a solution of liquid paraffin – *n*-hexane (1 + 2).

Morphine (*hR_f* 20–25), 6-monoacetylmorphine (*hR_f* 35–40) and heroin (*hR_f* 50–55) appeared as blue fluorescent zones on a dark background under long-

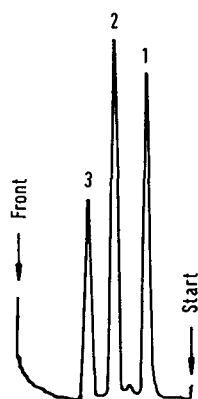


Fig. 1: Fluorescence scan of a mixture of alkaloids with ca. 50 ng substance per chromatogram zone: morphine (1), 6-monoacetylmorphine (2) and heroin (3).

wavelength UV light ($\lambda = 365$ nm). In each case the detection limits were 2 ng substance per chromatographic zone (Fig. 1).

In situ quantitation: The fluorimetric determination was carried out in UV light ($\lambda_{\text{exc}} = 313$ nm, $\lambda_{\text{fl}} > 390$ nm).

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Ammonium Thiocyanate – Iron(III) Chloride Reagent

Reagent for:

- Phosphates and phosphonic acids in detergents* [1]
 - Organic acids and phosphate esters [2]
- | | | |
|-----------------------|-------------------------|-----------------|
| | NH_4SCN | FeCl_3 |
| e.g. sugar phosphates | $M_r = 76.12$ | $M_r = 162.22$ |

Preparation of the Reagent

Dipping solution I Dissolve 1 g ammonium thiocyanate (ammonium rhodanide) in 100 ml acetone.

Dipping solution II Dissolve 50 mg iron(III) chloride in 100 ml acetone.

Storage The solutions are stable – when stored in the dark – for one month at room temperature [2].

Substances Ammonium thiocyanate
Iron(III) chloride anhydrous
Acetone

Reaction

Iron(III) thiocyanate is not formed to any extent in the chromatogram zones. The result is white zones on a pink-colored background:



Method

The chromatogram is dried for 10 min in a stream of warm air and immersed in solution I for 1 s. It is then dried for 5 min in a stream of warm air and finally immersed in solution II for 1 s. White zones result on a pink background.

Note: It is necessary to remove acid mobile phases completely, since the color reaction only occurs in neutral to weakly acid medium. This is often difficult when cellulose layers are employed so that interference can occur.

Some acids such as cinnamic, lactic, oxalic and quinaldic acid yield yellow zones. Maleic, fumaric and *o*-phthalic acids turn red in color, salicylic acid grey and 4-aminobenzoic acid bluish.

Dipping solution I alone is a sensitive detection reagent for phosphate esters. Combination with dipping solution II does increase the sensitivity limit for organic acids but it is still insufficient for sensitive detection. Inorganic ions that form complexes with iron(III) ions can interfere with detection. It is necessary to replace dipping solution II after each dipping procedure because accumulated impurities (e.g. NH_4SCN from I) can discolor the background.

The reagent can be employed on silica gel, kieselguhr, polyamide and cellulose layers. Only dipping solution I can be employed on amino phases.

Procedure Tested

Inorganic Phosphates and Phosphonic Acids in Detergents [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Cellulose (MERCK).
Mobile phase	Dioxan – trichloroacetic acid solution (70 + 30). <i>Preparation of the trichloroacetic acid solution:</i> 16 g trichloroacetic acid were dissolved in 50 ml water. 0.8 ml 33% ammonia solution was (carefully!) added (fume cupboard) and made up to 100 ml with water.

*) Rüdts, U.: Private communication, Chemische Untersuchungsanstalt, Stuttgart, 1984

Migration distance 6 cm

Running time 30 min

Detection and result: The chromatogram was dried for 10 min in a stream of warm air and immersed in solution I for 1 s, it was then dried for 5 min in a stream of warm air and immersed in solution II for 1 s.

The phosphates and phosphonic acids appeared as white zones on a pink background (Fig. 1A). Figure 1B is a reproduction of the reflectance plots ($\lambda = 480$ nm). Detection limits of 50 ng have been found for PO_4^{3-} and $\text{P}_2\text{O}_7^{4-}$. In the case of $\text{P}_3\text{O}_9^{3-}$ and $\text{P}_3\text{O}_{10}^{5-}$ the detection limits were 125 ng per chromatogram zone.

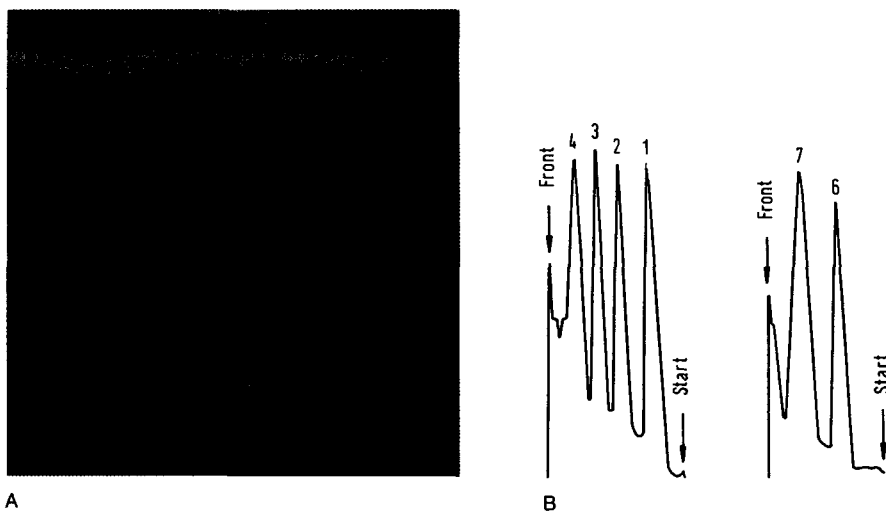


Fig. 1: A) Separation of phosphates and phosphonic acids; B) absorption plots of track 5 and track 8. $\text{P}_3\text{O}_9^{3-}$ (1), $\text{P}_3\text{O}_{10}^{5-}$ (2), $\text{P}_2\text{O}_7^{4-}$ (3), PO_4^{3-} (4), mixture I (5), aminotrimethylene-phosphonic acid (6), 1-hydroxyethane-1,1-diphosphonic acid (7), mixture II (8).

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Amylose – Potassium Iodate/Iodide Reagent

Reagent for:

- Nonvolatile aromatic and aliphatic carboxylic acids [1]

- Ampicillin [2]

- Phenylethylamines e.g. ephedrine [3]

KI	KIO ₃	Amylose
$M_r = 166.01$	$M_r = 214.00$	$M_r \approx 10^6$

Preparation of Reagent

Solution I Dissolve 1 g amylose with warming in 100 ml water.

Solution II Dissolve 2 g potassium iodate in 100 ml water.

Solution III Dissolve 8 g potassium iodide in 100 ml water.

Dipping solution Combine 10 ml each of solutions I to III immediately before use and dilute to 100 ml with water.

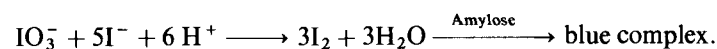
Spray solution Mix equal volumes of solutions I to III immediately before spraying.

Storage Solutions I to III are stable for several weeks in the refrigerator.

Substances Amylose
 Potassium iodide
 Potassium iodate

Reaction

Iodide and iodate ions react under the influence of protons to yield iodine molecules which react with amylose to yield a blue clathrate complex:



Method

The well-dried chromatogram (1 h at 105°C if acidic or basic eluents have been employed) is immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and then dried in a stream of cold air. Acids yield blue zones on a colorless or pale blue background [1] which gradually darkens.

For the detection of ampicillin it is necessary to add acetic acid to the dipping or spray solution. Ampicillin then yields pale zones on a blue background [2].

Note: The reagent can be employed on silica gel and cellulose layers. Starch can also be employed in place of amylose [2]. The blue coloration of the amylose complex turns brown after a short time.

Saccharin and the three diphenols, pyrocatechol, resorcinol and hydroquinone, react only weakly or not at all. The same is true of picric acid. On the other hand, cyclohexanesulfamic acid and bis-(2-ethylhexyl)-phosphoric acid are readily detected [1].

The detection limit for ampicillin is 50 ng per chromatogram zone.

Procedure Tested

Organic Acids [4]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Cellulose (MERCK). HPTLC plates Silica gel WRF _{254s} (MERCK). SIL G-25 (MACHEREY-NAGEL).
Mobile phase	a) for cellulose layers 1. 1-butanol — ethanol (96%) — ammonia solution (25%) — water (60 + 60 + 60 + 15)

2. ethanol (96%) — ammonia solution (25%) (112 + 16).

b) for M & N SIL G-25 and MERCK HPTLC plates

3. diisopropyl ether — formic acid — water (90 + 7 + 3).

Migration distance 6 cm

Running time 25–30 min

Detection and result: The chromatograms had to be freed from mobile phase before they were immersed; otherwise a blue background was produced. After it had been dipped the chromatogram was dried in a stream of cold air. Zones appearing on an initially pale background were first brown and then turned blue. The background, however, darkened so much that after 5 min it was scarcely possible to discern the zones. Table 1 lists some hR_f values.

Table 1: hR_f values of some carboxylic acids

Acid	hR_f value	
	Cellulose	Silica gel
4-Aminobenzoic acid	55–60	—
2-Aminobenzoic acid	60–65	—
Fumaric acid	—	80–85
Benzoic acid	70–75	—
Malic acid	—	15–20
Tartaric acid	5–10	0
Phthalic acid	15–20	60–65
Adipic acid	—	55–60
Salicylic acid	80–85	—

Note: The reagent was not particularly sensitive for acids. On cellulose layers the detection limit was ca. 1 µg (salicylic acid ≥ 5 µg) and on silica gel layers it was 5 µg (fumaric acid ca. 1 µg).

In situ quantitation: The reagent was not suitable for a sensitive, direct, photometric analysis.

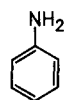
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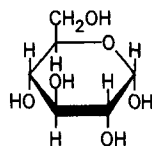
Aniline — Aldose Reagent

Reagent for:

- Organic acids [1–4]
- N-Acylglycine conjugates [7]



C_6H_7N
 $M_r = 93.13$
 Aniline



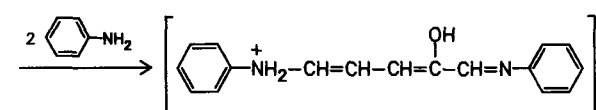
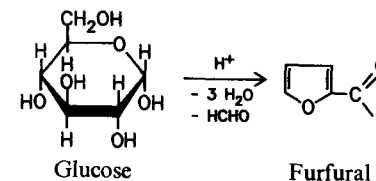
$C_6H_{12}O_6$
 $M_r = 180.16$
 Glucose

Preparation of the Reagent

Solution I	Mix 2 ml freshly distilled aniline with 18 ml ethanol.
Solution II	Dissolve 2 g of an aldose (e.g. glucose) in 20 ml water.
Dipping solution	Mix 20 ml each of solutions I and II immediately before use and make up to 100 ml with 1-butanol.
Storage	Solutions I and II are stable for a long period in the refrigerator.
Substances	D(+) Glucose Aniline Ethanol 1-Butanol

Reaction

Furfural is produced from glucose under the influence of acid and this reacts with aniline to yield a colored product [5].



Method

The chromatogram is freed from mobile phase and immersed in the dipping solution for 3 s or the solution is sprayed on homogeneously; the chromatogram is then heated to 90–140°C for 5–10 min. Brown zones are produced on a beige-grey background.

Note: Aldoses other than glucose can also be used e.g. arabinose [1], xylose [2, 3, 7] or ribose [4]. The background color is least on cellulose layers; when cellulose acetate, aluminium oxide 150, silica gel, RP, NH₂ or polyamide layers are employed the background is a more or less intense ochre. The detection limit of carboxylic acids on cellulose layers is ca. 0.5 µg substance per chromatogram zone.

It has not been possible to ascertain why this reagent is occasionally referred to as SCHWEPPE reagent.

Procedure Tested

Organic Acids [6]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Sil G-25 (MACHEREY-NAGEL).

Mobile phase Diisopropyl ether — formic acid — water (90 + 7 + 3).

Migration distance 10 cm

Running time 30 min

Detection and result: The chromatogram was freed from mobile phase and immersed for 3 s in the dipping solution and heated to 125°C for 5–10 min. The carboxylic acids: terephthalic acid (hR_f 5), succinic acid (hR_f 50–55), phthalic acid (hR_f 55–60), suberic acid (hR_f 60–65), sebacic acid (hR_f 65–70), benzoic acid (hR_f 75–80) and salicylic acid (hR_f 80–85) yielded brown zones on a light brown background. The detection limit was 2 µg acid per chromatogram zone.

Note: If a dipping solution was employed for detection whose concentration was reduced to 1/10th that given above the acids appeared as white zones on a light brown background.

In situ quantitation: The reagent was not suitable for a sensitive, direct, photometric analysis.

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Aniline — Diphenylamine — Phosphoric Acid Reagent

Reagent for:

- Sugars
Mono- and disaccharides
[1–5]

Oligosaccharides [5–7]

Starch hydrolysates [8–12]

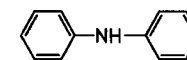
- Thickening agents [13, 14]

- Glycosides [15, 18]
e.g. arbutin, prunasin,
amygdalin, rutin



C_6H_7N

$M_r = 93.13$



$C_{12}H_{11}N$

$M_r = 169.23$

H_3PO_4

$M_r = 98.00$

Diphenyl-
amine

Orthophos-
phoric acid

Preparation of the Reagent

Dipping solution Dissolve 2 g diphenylamine and 2 ml aniline in 80 ml acetone. Carefully add 15 ml phosphoric acid and make up to 100 ml with acetone [2, 8].

Spray solution Dissolve 1 to 2 g diphenylamine and 1 to 2 ml aniline in 80 ml methanol or ethanol. After addition of 10 ml phosphoric acid make up to 100 ml with methanol [3, 9] or ethanol [7].

Storage Both reagents can be stored in the dark at 4°C for up to 14 days. It is recommended that the reagent be prepared daily for in situ quantitation.

Substances Diphenylamine
Aniline
Orthophosphoric acid

Procedure Tested

Glucose, Fructose, Maltose or Lactose, Sucrose and Raffinose [17]

Two noninterchangeable methods of procedure are reported below under the headings A and B.

Method	A) Ascending, one-dimensional, double development in a trough chamber (5 min drying in warm air between developments) with chamber saturation. B) Ascending, one-dimensional development at 20°C in a trough chamber.
Layer	A) HPTLC plates Si 50000 (MERCK); develop once in chloroform — methanol (1 + 1) to prewash and then dry at 110°C for 30 min. B) HPTLC plates NH ₂ F _{254s} (MERCK).
Mobile phase	A) Acetonitrile — water (17 + 3). B) Acetonitrile — water — phosphate buffer (16 + 3 + 2) <i>Preparation of the phosphate buffer:</i> Make up 10 ml of a mixture of 50 ml potassium dihydrogen phosphate solution (<i>c</i> = 0.1 mol/l) and 4.6 ml caustic soda solution (<i>c</i> = 0.1 mol/l) to 100 ml with water.
Migration distance	A) 2 × 7 cm B) 7 cm
Running time	A) 2 × 10 min B) 15 — 20 min

Detection and result: A) and B): The chromatogram was dried for 3 min in a stream of warm air, immersed in the dipping solution for 9 s and then heated to 105–110°C for 15 min and finally immersed for 2 s in a solution of liquid paraffin—*n*-hexane (1 + 2) to stabilize and increase the fluorescence intensity (factor 2 to 3).

Grey-green zones on a white background resulted; they exhibited weak red fluorescence under long-wavelength UV light ($\lambda = 365$ nm); glucose and fructose exhibited pale blue fluorescence in method B. Some hR_f values are listed in Table 1.

Table 1: hR_f values of some sugars

Sugar	hR_f value	
	Method A	Method B
Fructose	45–50	25–30
Glucose	35–40	20–25
Sucrose	25–30	15–20
Lactose	15–20	—
Maltose	—	10–15
Raffinose	10–15	5–10

In situ quantitation: Quantitative analysis (Figs. 2 and 3) could be performed both absorption-photometrically with long-wavelength UV light ($\lambda = 365$ nm) or fluorimetrically ($\lambda_{exc} = 436$ nm; $\lambda_{fl} = 546$ nm [monochromation filter M 546] or $\lambda_{fl} > 560$ nm).

The detection limit for fluorimetric quantitation was 10 ng substance per chromatogram zone.

Note: If the chromatogram developed by method B was exposed to ammonia vapors for 10 min before being immersed in liquid paraffin—*n*-hexane (1 + 2) the fluorescence of the chromatogram zones became deep red. Glucose and fructose also appeared red.

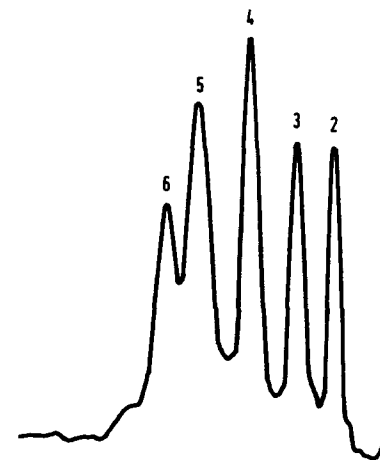


Fig. 2: Fluorescence plots of the sugars after separation on a Si-50 000 layer without ammonia-vapor treatment. Start (1), raffinose (2), lactose (3), sucrose (4), glucose (5), fructose (6).

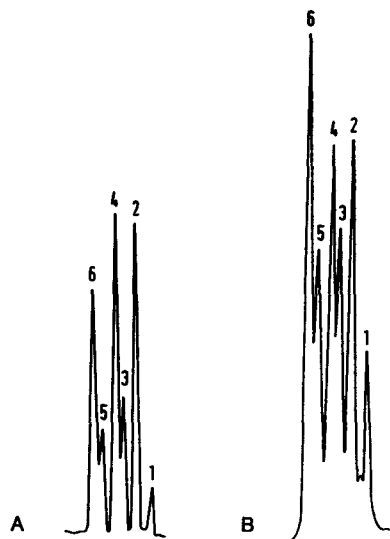


Fig. 3: Absorbance plots (A) and fluorescence plots of the sugars without ammonia-vapor treatment (B) after separation on a NH_2 layer. Start (1), raffinose (2), maltose (3), sucrose (4), glucose (5), fructose (6).

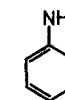
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Aniline – Phosphoric Acid Reagent

Reagent for:

- Carbohydrates (sugars)
e.g. monosaccharides [1]
- Glucosides
e.g. aryl- and thioglucosides [2]



$\text{C}_6\text{H}_7\text{N}$
 $M_r = 93.13$
Aniline

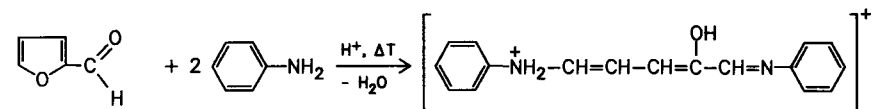
H_3PO_4
 $M_r = 98.00$
Orthophosphoric
acid

Preparation of the Reagent

- Solution I** Make 15 ml aniline up to 100 ml with 1-butanol in a volumetric flask.
- Solution II** Carefully make 30 ml orthophosphoric acid (88–90%) up to 100 ml with 1-butanol in a volumetric flask.
- Dipping solution** Immediately before use add 20 ml solution I to 50 ml solution II and mix well to redissolve the precipitate that is produced. If this does not go back into solution it should be filtered off.
- Storage** Solutions I and II are stable for several days.
- Substances** Aniline
Orthophosphoric acid
1-Butanol

Reaction (after [3])

Heating the sugars with strong acid yields furfural derivatives. Under these conditions aldohexoses can eliminate formaldehyde and water to yield furfural. This aldehyde reacts with amines to yield colored SCHIFF's bases.



Method

The chromatogram is freed from mobile phase in the drying cupboard (10 min, 120°C) and immersed for 1 s in the reagent solution or sprayed homogeneously with it until the plate starts to appear transparent; it is then dried briefly in a stream of warm air and heated to 125–130°C for 45 min.

Many aryl- and also thio-β-D-glucosides produce yellow fluorescent chromatogram zones on a dark violet background under long-wavelength UV light ($\lambda = 365$ nm), the zones are also sometimes recognizable in visible light as grey-brown zones on a white background [2]. Monosaccharides produce dark brown chromatogram zones on a white background [1].

Note: The detection limit for aryl- and thioglucosides is 100–200 ng substance per chromatogram zone [2]. Reduction of the proportion of phosphoric acid in the reagent leads to loss of sensitivity [2].

The reagent can be employed on silica gel, RP-18, CN and NH₂ layers. Cellulose and polyamide layers are not suitable.

Procedure Tested

Thioglucosides [4]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	1-Butanol – 1-propanol – glacial acetic acid – water (30 + 10 + 10 + 10).
Migration distance	5 cm
Running time	45 min

Detection and result: The chromatogram was freed from mobile phase in the drying cupboard (10 min, 125°C) and immersed for 1 s in the reagent solution, then heated to 120°C for 45 min.

Sinigrin (*hR_f* 35–40) appeared as a yellow fluorescent chromatogram zone on a dark background under long-wavelength UV light ($\lambda = 365$ nm). The detection limit was 25–50 ng substance per chromatogram zone.

In situ quantitation: Excitation at $\lambda_{\text{exc}} = 365$ nm and measurement at $\lambda_{\text{fl}} > 560$ nm were employed for fluorimetric quantitation (Fig. 1).

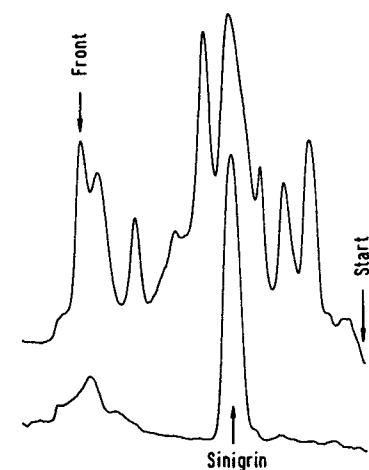


Fig. 1: Fluorescence plot (A) of the chromatogram track of an “unpurified” extract of sinapis seed (application: 2 µl of a 1% solution in methanol) and (B) of a reference track with 1 µg sinigrin per chromatogram zone.

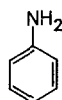
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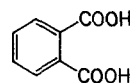
Aniline – Phthalic Acid Reagent (Aniline phthalate Reagent)

Reagent for:

- Halogen oxyacids [1]
e.g. chlorate, chlorite, perchlorate, bromate, bromite, iodate
- Reducing carbohydrates (sugars)
e.g. monosaccharides [2–8]
oligosaccharides [2, 8]
oligouronic acids [9]
methyl sugars [10, 11]



$C_6H_5NH_2$
 $M_r = 93.13$
Aniline



$C_8H_6O_4$
 $M_r = 166.13$
Phthalic acid

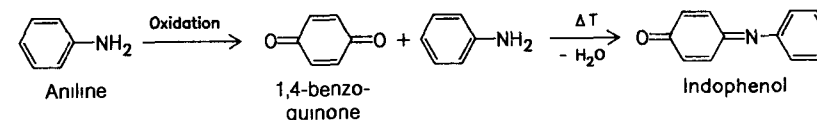
Preparation of Reagent

Dipping solution	Dissolve 0.9 ml aniline and 1.66 g phthalic acid in 100 ml acetone.
Spray solution	Dissolve 0.9 ml aniline and 1.66 g phthalic acid in 100 ml water-saturated 1-butanol [1].
Storage	The reagent solutions may be stored for several days.
Substances	Aniline Phthalic acid 1-Butanol Acetone

Reaction (according to [12])

Furfural derivatives are produced when sugars are heated with acids (see Aniline – Diphenylamine – Phosphoric Acid Reagent), these condense with aniline to SCHIFF's

bases. Chlorate, chlorite, perchlorate etc. oxidize aniline to 1,4-quinone, which then reacts with excess aniline to yield indophenol:



Method

The chromatograms are freed from mobile phase, immersed in the reagent solution for 1 s or homogeneously sprayed with it, dried briefly in a stream of warm air and heated to 80–130°C for 20–30 min.

Variously colored chromatogram zones are formed on an almost colorless background [1, 4], some of which fluoresce after irradiation with long-wavelength UV light ($\lambda = 365 \text{ nm}$) [5].

Note: The dipping solution can also be employed as spray reagent. The detection limits per chromatogram zone are reported to be 1–5 μg substance [1] for the oxyacids of halogens and ca. 10 μg substance for reducing sugars [4].

The reagent may be employed on silica gel, kieselguhr, aluminium oxide, cellulose and polyamide layers.

Procedure Tested

Halogen Acids and Halogen Oxyacids [13]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	2-Propanol – tetrahydrofuran – ammonia solution (32%) (50 + 30 + 20).
Migration distance	5 cm
Running time	25 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in the reagent solution for 1 s and heated to 120°C for 20 min. Intense yellow to brown zones of various hues were produced; these appeared as dark zones on a fluorescent background under long-wavelength UV light ($\lambda = 365$ nm).

While chlorate and bromate yielded strong brown zones, iodate did not react at all. Iodide yielded an intense yellow zone, the colors produced by chloride, bromide and perchlorate were weak (Fig. 1).

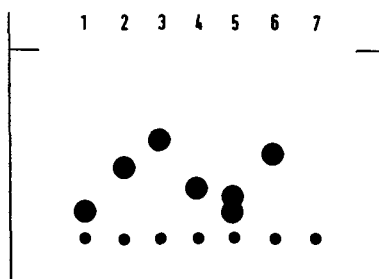


Fig. 1: Schematic diagram of a chromatogram of halogen acids and halogen oxyacids: Chloride (1), chlorate (2), perchlorate (3), bromide (4), bromate (5), iodide (6), iodate (7).

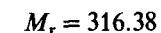
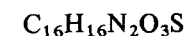
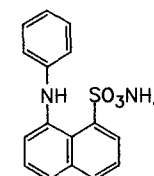
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8-Anilinonaphthalene-1-sulfonic Acid Ammonium Salt Reagent (ANS Reagent)

Reagent for:

- Lipids, phospholipids [1-8]
e.g. fatty acids or their methylesters,
fatty alcohols [9]
- Cholesterol and cholesteryl esters [1, 2]
- Steroids [3]
- Detergents [3]
- Hydrocarbons [3]



Preparation of the Reagent

- | | |
|-------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Dipping solution | Dissolve 100 mg of 8-anilinonaphthalene-1-sulfonic acid ammonium salt in a mixture of 40 ml caustic soda solution ($c = 0.1$ mol/l) and 57 ml of an aqueous solution containing 21 g citric acid monohydrate and 8 g sodium hydroxide per liter. |
| Spray solution | Dissolve 100 mg of 8-anilinonaphthalene-1-sulfonic acid ammonium salt in 100 ml water [1]. |
| Storage | The solutions are stable for at least 3 months if stored in a refrigerator in the dark [3]. |
| Substances | 8-Anilinonaphthalene-1-sulfonic acid
ammonium salt
Citric acid monohydrate
Sodium hydroxide pellets
Caustic soda (0.1 mol/l) |

Reaction

8-Anilinonaphthalene-1-sulfonic acid ammonium salt, which scarcely fluoresces in aqueous solution, is stimulated to intense fluorescence by long-wavelength UV light ($\lambda = 365$ nm) if it is dissolved in nonpolar solvents or adsorptively bound to nonpolar molecular regions [3].

Method

The developed chromatogram is freed from mobile phase by heating to 110°C for 10 min in the drying cupboard. It is allowed to cool and immersed for 1 s in or sprayed homogeneously with the reagent; the plate is then examined (while still moist).

Yellow-green fluorescent zones are easily visible against a dark background under long-wavelength UV light ($\lambda = 365$ nm).

Note: The developed chromatogram must be completely freed from nonpolar solvents before derivatization, otherwise an intense fluorescence will be stimulated over the whole plate. The fluorescence intensity of the chromatogram zones remains stable for ca. 40 min; it decreases slowly as the layer dries out and can be returned to its original intensity by renewed immersion in the reagent solution or in water.

The reagent can be employed on silica gel, kieselguhr and Si 50 000 layers and, if necessary, on RP-2 and RP-8 phases. It cannot be used on RP-18 layers [9] because here the whole plate is fluorescent. *)

Procedure Tested

Cholesterol [2]

Method Ascending, one-dimensional development in a twin-trough chamber.

Layer	HPTLC plates Silica gel 60 WRF _{245s} (MERCK). After application of the samples the plate was preconditioned for 30 min at 0% relative humidity.
Mobile phase	Cyclohexane – diethyl ether (1 + 1).
Migration distance	6 cm
Running time	15 min

Detection and result: The developed chromatogram was freed from mobile phase by drying for 10 min at 110°C , allowed to cool and immersed for 1 s in the reagent solution. The plate was evaluated as rapidly as possible while it was moist since the fluorescent background increased in intensity as the plate dried out. Cholesterol appeared as a yellow-green fluorescent zone (R_f 20–25).

In situ quantitation: Fluorimetric analysis was made with long-wavelength UV light ($\lambda_{\text{exc}} = 365$ nm, $\lambda_{\text{fl}} > 430$ nm). The detection limit on HPTLC plates that were analyzed in a moist state was 25 ng cholesterol per chromatogram zone (Fig. 1).

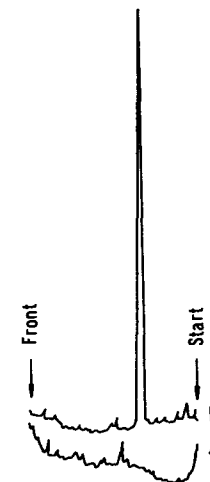


Fig. 1: Fluorescence plot of a blank track (A) and of a cholesterol standard with 200 ng substance per chromatogram zone (B).

*) Jork, H.: Private communication, Universität des Saarlandes, 66 Saarbrücken, 1987

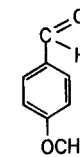
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Anisaldehyde – Sulfuric Acid Reagent

Reagent for:

- Antioxidants [1]
- Steroids [2 – 4]
e.g. estrogens [2, 3], androgens [3]
sterols, bile acids [3]
- Prostaglandins [5]
- Carbohydrates (sugars) [4, 6]
- Phenols [4, 7]
e.g. salicyl alcohol, salicylsalicin [8]
- Glycosides
e.g. cardiac glycosides [4, 12]
diterpene glycosides [17]
- Sapogenins
e.g. polygalaic acid [8], diosgenin
tigogenin, gitogenin [9]
- Essential oil components or
terpenes [4, 10, 11]
e.g. from *Hedeoma pulegioides* [10],
Melissae folium [11]
- Antibiotics [13, 14]
e.g. macrolide antibiotics [13]
heptaene antibiotics [14]
tetracyclines [15]
- Mycotoxins (trichothecenes) [16]



$C_8H_8O_2$
 $M_r = 136.15$

H_2SO_4
 $M_r = 98.08$

Anisaldehyde Sulfuric acid

Preparation of the Reagent

Dipping solution	Dissolve 1 ml 4-methoxybenzaldehyde (anisaldehyde) and 2 ml conc. sulfuric acid in 100 ml glacial acetic acid.
Spray solution	Carefully add 8 ml conc. sulfuric acid and 0.5 ml anisaldehyde under cooling with ice to a mixture of 85 ml methanol and 10 ml glacial acetic acid [1, 4, 8, 11, 16].
Storage	The reagents are stable for several weeks in the refrigerator [4].
Substances	4-Methoxybenzaldehyde Sulfuric acid Acetic acid Methanol Ethanol

Reaction

The mechanism of reaction with steroids has not been elucidated. Various nonquantitative reactions occur simultaneously. Cyclopentenyl cations have been postulated as intermediates which condense with anisaldehyde to yield colored compounds [4]. It is probable that triphenylmethane dyes are also formed with aromatic compounds.

Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed for 1 s in the dipping solution or sprayed homogeneously with the spray solution until the layer begins to become transparent and then heated to 90–125°C for 1–15 min.

Variously colored chromatogram zones result on an almost colorless background, they are often fluorescent under long-wavelength UV light ($\lambda = 365$ nm) (e.g. prostaglandins, salicylsalicycin).

Note: Anisaldehyde — sulfuric acid is a universal reagent for natural products, that makes color differentiation possible [6]. The background acquires a reddish coloration if the heating is carried out for too long; it can be decolorized again by interaction with water vapor. The dipping solution can be modified by the addition of *n*-hexane for the detection of glycosides [17]. The detection limits are 50 ng substance per chromatogram zone for prostaglandins and sugars [5, 6]. Bacitracin, chloramphenicol and penicillin do not react [15].

The reagent can be employed on silica gel, kieselguhr, Si 50 000 and RP layers.

Procedure Tested

Essential Oils [18]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene — chloroform (10 + 10).
Migration distance	2 × 6 cm with intermediate drying in a stream of cold air.
Running time	2 × 10 min

Detection and result: The chromatogram was freed from mobile phase and immersed for 1 s in the dipping solution and then heated to 100°C for 10 min.

Menthol (*hR_f* 15) and menthyl acetate (*hR_f* 55) yielded blue chromatogram zones; caryophyllene (*hR_f* 90) and caryophyllene epoxide (*hR_f* 20–25) appeared red-violet and thymol (*hR_f* 40–45) appeared brick red in color.

In situ quantitation: Absorption photometric recording in reflectance was performed at a medium wavelength $\lambda = 500$ nm (Fig. 1).

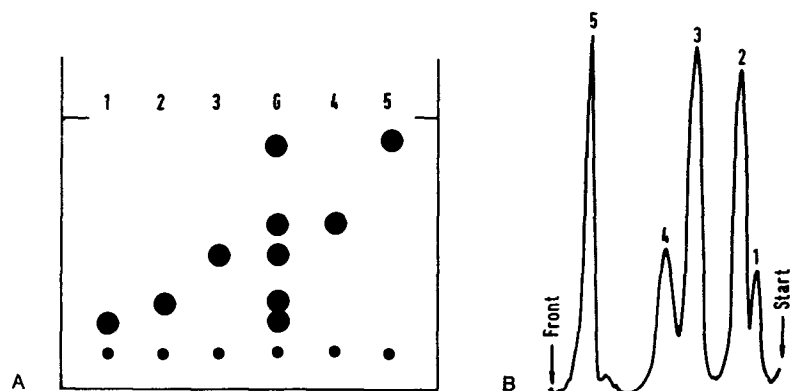


Fig. 1: Schematic sketch (A) of the separation of essential oil components (ca. 500 ng of each component) and reflectance scan of the mixture (B). Menthol (1), caryophyllene epoxide (2), thymol (3), menthyl acetate (4), caryophyllene (5), mixture (G).

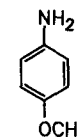
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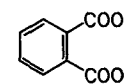
p-Anisidine – Phthalic Acid Reagent

Reagent for:

- Carbohydrates
e.g. monosaccharides [1–7, 12]
oligosaccharides [2, 6]
uronic acids [1, 4, 6]



C_7H_9NO
 $M_r = 123.16$
p-Anisidine



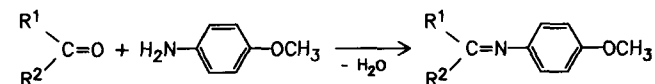
$C_8H_6O_4$
 $M_r = 166.13$
Phthalic acid

Preparation of the Reagent

- Solution I** Dissolve 1.25 g *p*-anisidine (4-methoxyaniline) in 25 ml methanol and add 25 ml ethyl acetate.
- Solution II** Dissolve 1.5 g phthalic acid in 25 ml methanol and dilute with 25 ml ethyl acetate.
- Dipping solution** Mix equal quantities of solutions I and II immediately before dipping.
- Storage** The two solutions I and II may be stored for several weeks in the refrigerator.
- Substances** *p*-Anisidine
Phthalic acid
Methanol
Ethyl acetate

Reaction

Sugars react with the reagent probably with the formation of SCHIFF's bases:



Method

The chromatogram is freed from mobile phase and immersed in the dipping solution for 1 s or uniformly sprayed with it and then heated to 100–130°C for 10 min. The result is reddish-brown (pentoses) to brown chromatogram zones on a colorless background, which also becomes brown after a time.

Note: Phosphoric acid [8] and hydrochloric acid [6, 9] have both been suggested in the literature as substitutes for phthalic acid. The addition of sodium dithionite [9] is also occasionally mentioned and sometimes no additives are employed [10]. The alternative reagents offer no advantages over the phthalic acid containing reagent since they usually cause more background coloration. The limits of detection are about 0.1–0.5 µg per chromatogram zone [5].

The reagent can be employed on silica gel, RP, NH₂ and polyamide layers.

Procedure Tested

Monosaccharides [11]

Method	Ascending, one-dimensional double development in a HPTLC trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	2-Propanol — boric acid (2% aqueous solution) — glacial acetic acid (40 + 5 + 1).
Migration distance	2 × 8 cm
Running time	2 × 90 min

Detection and result: The chromatogram was freed from mobile phase and immersed in the reagent solution for 1 s and then heated to 130°C for 10 min. Rhamnose (*hR_f* 35–40) and fructose (*hR_f* 70–75) yielded brown and xylose (*hR_f* 45–50) and arabinose (*hR_f* 60–65) red-brown chromatogram zones on a pale background. The detection limit for the pentoses was 0.1 µg and for fructose it was 0.5 µg substance per chromatogram zone.

In situ quantitation: Absorption photometric scanning was carried out in reflectance at $\lambda = 480$ nm (Fig. 1).

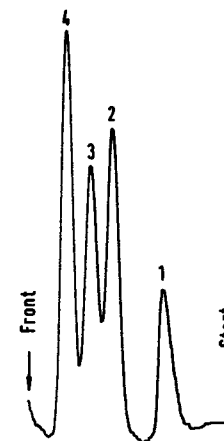


Fig. 1: Absorption curve of a chromatogram track with 4 µg of each substance per chromatogram zone. Rhamnose (1), xylose (2), arabinose (3), fructose (4).

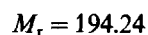
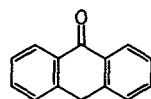
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Anthrone Reagent

Reagent for:

- Ketoses [1]
- Glycolipids, gangliosides [2–7]
- Cyclodextrins [8]



Preparation of Reagent

Dipping solution For ketoses: Dissolve 300 mg anthrone in 10 ml acetic acid and add in order 20 ml ethanol, 3 ml 85% phosphoric acid and 1 ml water [1].

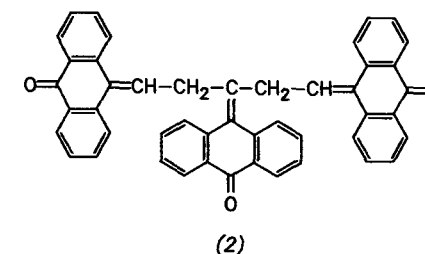
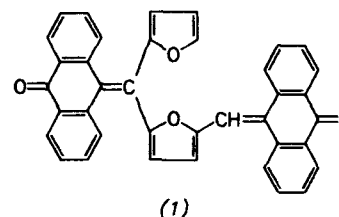
Spray solution For glycolipids: Carefully add 72 ml conc. sulfuric acid to 28 ml water with cooling. Dissolve 50 mg anthrone in the mixture with gentle warming [5].

Storage The dipping solution may be stored in the refrigerator for several weeks, the spray solution should be freshly prepared each day.

Substances Anthrone
Sulfuric acid (95–97%)
Acetic acid (96%)
Ethanol
Orthophosphoric acid (85%)

Reaction

Carbonyl derivatives react with anthrone in acidic medium to yield condensation products of types 1 (pentoses) or 2 (hexoses) [9]:



Method

The chromatogram is freed from mobile phase in a stream of warm air and immersed for 4 s in the dipping solution or evenly sprayed with it until the layer begins to be transparent (the spray solution is employed for glycosides) and then heated to 105–120°C for 5–15 min.

Colored chromatogram zones appear on an almost colorless background; ketoses, for example, are yellow in color [1].

Note: Aldoses do not react or only react with greatly reduced sensitivity. The reagent can be employed with silica gel, kieselguhr and Si 50 000 layers. Paraffin-impregnated silica gel layers may also be employed [8].

Procedure Tested

Raffinose, Sucrose, Fructose [10]

Method Ascending, one-dimensional double development in a trough chamber with chamber saturation.

Layer HPTLC plates Si 50 000 (MERCK); before application of the samples the layer was developed once in chloroform–methanol (50 + 50) to precleanse it and dried at 110°C for 30 min.

Mobile phase Acetonitrile – water (85 + 15).

Migration distance 2 × 7 cm with 5 min intermediate drying in a stream of warm air.

Running time 2 × 10 min

Detection and result: The chromatogram was dried for 5 min in a stream of warm air, immersed in the reagent for 4 s and then heated to 110 °C for 8 min. After cooling to room temperature it was immersed for 2 s in a 20% solution of dioctyl-sulfosuccinate in chloroform.

In visible light raffinose (hR_f 10–15), sucrose (hR_f 30–35) and fructose (hR_f 45–50) produced yellow zones on a light background, in long-wavelength UV light ($\lambda = 365$ nm) the zones had a red fluorescence on a pale blue background. The detection limits were less than 10 ng substance per chromatogram zone (Fig. 1).

In situ quantitation: The absorption photometric scan in reflectance was made at $\lambda = 435$ nm (detection limit 20–30 ng per chromatogram zone). Fluorimetric scanning was performed at $\lambda_{exc} = 436$ nm and $\lambda_{fl} > 560$ nm (detection limit < 10 ng per chromatogram zone).

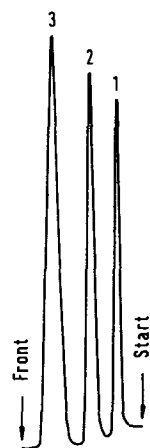


Fig. 1: Fluorescence scan of a chromatogram track with 100 ng substance per chromatogram zone. Raffinose (1), sucrose (2), fructose (3).

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Antimony(III) Chloride Reagent (Carr-Price Reagent)

Reagent for:

- Vitamins A and D [1, 2]
- Carotinoids [1, 2]
- Terpenes, triterpenes [1 – 5]
- Sterols [6]
e.g. β -sitosterol [7]
- Steroids, steroid hormones [8 – 10]
e.g. contraceptives [10]
steroid alkaloids [11]
- Bile acids [12]
- Sapogenins, steroid sapogenins [2, 13 – 16]
- Glycosides [1]
e.g. steroid glycosides [2]
digitalis glycosides [17, 18]
- Flavonoids [3]
- Phospholipids [19]

SbCl₃ $M_r = 228.11$

Preparation of Reagent

Dipping solution Dissolve 2 g antimony(III) chloride in 50 ml chloroform.

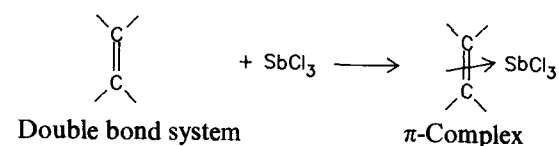
Spray solution Dissolve 10 g antimony(III) chloride in 50 ml chloroform or carbon tetrachloride.

Storage The solution should always be freshly made up.

Substances Antimony(III) chloride
Chloroform
Carbon tetrachloride

Reaction

Antimony(III) chloride forms colored π -complexes with double bond systems (e.g. vitamin A).



Method

The chromatograms are freed from mobile phase, immersed in the dipping solution for 1 s or sprayed evenly with the spray solution and then heated to 110 – 120°C for 5 – 10 min.

Variously colored chromatogram zones are sometimes produced even before heating; they often fluoresce in long-wavelength UV light ($\lambda = 365$ nm)

Note: The reagent is also occasionally employed in hydrochloric acid [5, 14 – 16] or acetic acid [10] solution or with the addition of acetic anhydride [7] or sulfuric acid [12]. The solvents employed should be as anhydrous as possible.

The reagent can be employed on silica gel, kieselguhr, Si 50 000 and aluminium oxide layers.

Procedure Tested

Vitamin D₃ [20]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK). Before sample application the layers were prewashed by developing once with chloroform – methanol (50 + 50) and dried at 110°C for 30 min.
Mobile phase	Cyclohexane – diethyl ether (40 + 20).
Migration distance	6 cm
Running time	15 min

Detection and result: The developed chromatogram was dried in a stream of cold air for 15 min, then immersed in the dipping solution for 1 s and, after brief drying in a stream of warm air, heated to 110°C for 10 min. Vitamin D₃ (R_f 15–20, detection limit ca. 1 ng per chromatogram zone) appeared in visible light as a grey chromatogram zone on a white background and in long-wavelength UV light ($\lambda = 365$ nm) as a bright red fluorescent zone on a blue background.

The fluorescence intensity was stabilized and enhanced (ca. 2-fold) by immersion of the chromatogram for 1 s in a mixture of liquid paraffin – *n*-hexane (1 + 2).

In situ quantitation: The fluorimetric analysis was made at $\lambda_{exc} = 436$ nm and $\lambda_{fl} > 560$ nm (Fig. 1).

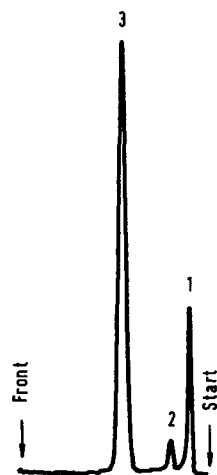


Fig. 1: Fluorescence scan of a chromatogram track with 50 ng vitamin D₃ per chromatogram zone: by-products (1, 2), vitamin D₃ (3).

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Antimony(V) Chloride Reagent

Reagent for:

- Esters of phenoxyalkancarboxylic acids
e.g herbicides [1]
- Components of essential oils [2–4]
- Terpenes, triterpenes [2, 5]
- Steroids [6, 7]
- Phenols, phenol ethers [2, 8, 9]
- Antioxidants [9]
- Aromatic hydrocarbons [10]



$$M_r = 299.02$$

Preparation of Reagent

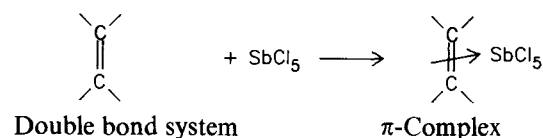
Dipping solution Mix 2 ml antimony(V) chloride with 8 ml carbon tetrachloride.

Storage The reagent solution should always be freshly prepared.

Substances Antimony(V) chloride
Carbon tetrachloride

Reaction

Antimony(V) chloride forms colored π -complexes with double bond systems.



Method

The chromatograms are freed from mobile phase, immersed in the dipping solution for 1 s or homogeneously sprayed with it until they begin to be transparent and then heated to 105–120° for 5–10 min.

Various colored chromatogram zones are produced on a colorless background; some of them fluoresce under long-wavelength UV light ($\lambda = 365$ nm).

Note: The solvents employed should be anhydrous. The esters of phenoxyalkancarboxylic acids (detection limits: 500 ng) [1] yield brown to violet, terpenes violet-grey [2] and triterpenes yellow to violet [5] colored chromatogram zones.

The reagent can be employed on silica gel, kieselguhr, Si 50 000 and aluminium oxide layers.

Procedure Tested

Essential Oil of Peppermint

Method	Ascending, one-dimensional development in a HPTLC trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Dichloromethane.
Migration distance	5 cm
Running time	8 min

Detection and result: The chromatograms were dried in a stream of warm air, immersed for 1 s in the reagent solution and dried at 120°C for 5 min. Menthol (hR_f 15–20), cineole (hR_f 20–25), menthone (hR_f 35–40), menthyl acetate (hR_f 45–50) and menthofuran (hR_f 80–85) yielded grey to brown chromatogram zones on a pale background, they fluoresced yellow under long-wavelength UV light ($\lambda = 365$ nm). The detection limit for menthol was 15 ng substance per chromatogram zone (Fig. 1).

In situ quantitation: The fluorimetric analysis was carried out at $\lambda_{\text{exc}} = 365$ nm and $\lambda_{\text{fl}} > 560$ nm (Fig. 2).

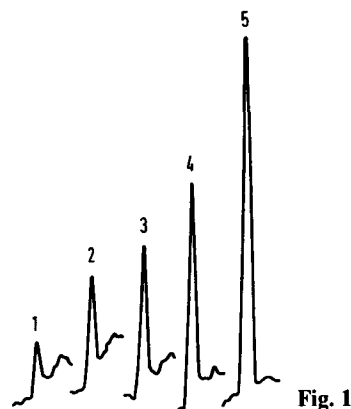


Fig. 1: Fluorescence scans of a series of dilutions for evaluation of the detection limit of menthol: 15 ng (1), 30 ng (2), 75 ng (3), 150 ng (4), 300 ng (5).

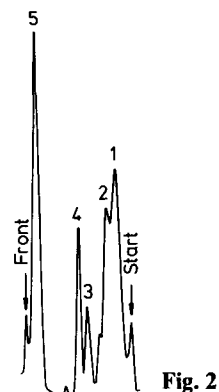


Fig. 2: Fluorescence scan of a chromatogram of peppermint oil: menthol (1), cineole (2), menthone (3), menthyl acetate (4), menthofuran/terpenes (5).

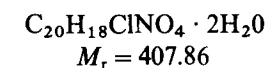
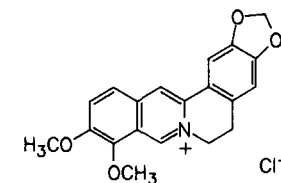
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Berberine Reagent

Reagent for:

- Sterols [1–3]
e.g. sitosterol, stigmasterol,
campesterol, cholesterol
- Saturated compounds [4]



Preparation of Reagent

Dipping solution Dissolve 10 mg berberine chloride in 100 ml ethanol.

Storage The dipping solution may be kept for several days.

Substances Berberine chloride
Ethanol

Reaction

The mechanism of the reaction has not been elucidated. Berberine is probably enriched in the lipophilic chromatogram zones which then fluoresce more intensely than the environment.

Method

The chromatograms are freed from mobile phase, immersed in the reagent solution for 1 s or sprayed homogeneously with it and then dried in a stream of cold air.

Under UV light ($\lambda = 254$ nm or 365 nm) the chromatogram zones appear as pale yellow fluorescent zones on a weakly yellow fluorescent background.

Note: The reagent can also be applied before chromatography e.g. by impregnating the layer, which should preferably be free from fluorescent indicator; it is not eluted by most mobile phases [4].

The reagent can be employed on silica gel, silver nitrate-impregnated silica gel, carboxymethylcellulose-containing silica gel, kieselguhr and Si 50000 layers; RP phases are not suitable.

Procedure Tested

Sterols, Fatty Acids, Triglycerides, Hydrocarbons [5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	<i>n</i> -Hexane – diethyl ether – glacial acetic acid (80 + 20 + 1).
Migration distance	5 cm
Running time	7 min

Detection and result: The chromatogram was dried in a stream of cold air for 5 min and immersed in the dipping solution for 1 s. Cholesterol (*hR_f* 10–15),

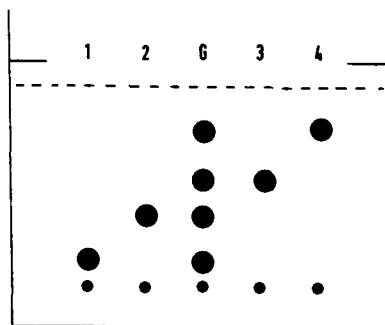


Fig. 1: Sketch of the chromatographic separation of a mixture (G) of cholesterol (1), stearic acid (2), tripalmitin (3) and caryophyllene (4).

stearic acid (*hR_f* 20–25), tripalmitin (*hR_f* 45–50) and caryophyllene (*hR_f* 90–95) appeared as pale yellow fluorescent zones on a less intense yellow fluorescent background. The visual detection limits per chromatogram zone were 10 ng for cholesterol, 50 ng for stearic acid and tripalmitin and 100 ng for caryophyllene (Fig. 1).

In situ quantitation: The reagent is unsuitable for in situ quantitation.

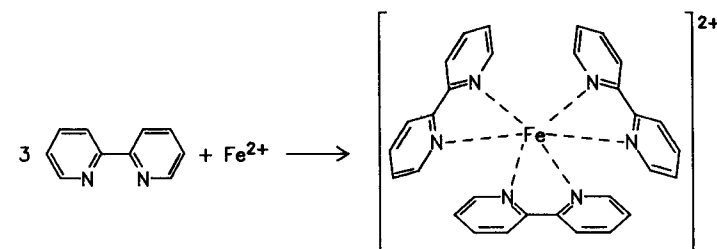
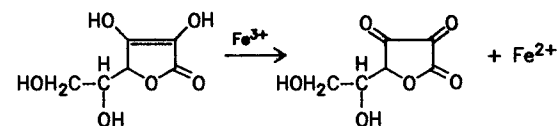
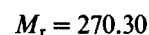
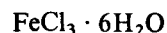
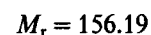
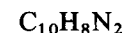
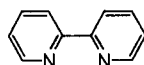
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2,2'-Bipyridine — Iron(III) Chloride Reagent (Emmerie-Engel Reagent)

Reagent for:

- Phenols [1]
- Vitamin E (tocopherols) [2–4]
- Antioxidants [5]
- Reducing substances
e.g. ascorbic acid [6]



Method

The chromatogram is freed from mobile phase in a stream of warm air and immersed in the reagent solution for 1 s and then dried in a stream of cold air. Red to reddish-brown zones are formed on a colorless background. They often appear immediately but sometimes they only appear after some minutes; their color intensity is completely developed after 30 min [1]. They can be employed for quantitative analysis.

Note: The dipping reagent, which can also be applied as a spray reagent, can be employed on cellulose and silica gel layers. A 3% solution of 2,2'-bipyridine in 40% thioglycolic acid can be employed as a specific spray reagent for the detection of iron (red coloration) [7].

Procedure Tested

α -Tocopherol [3]

Method Horizontal, one-dimensional development in a linear chamber (CAMAG).

Layer HPTLC plates Silica gel 60 F₂₅₄ (MERCK).

Preparation of the Reagent

Solution I Dissolve 0.1 g iron(III) chloride hexahydrate in 50 ml ethanol.

Solution II Dissolve 0.25 g 2,2'-bipyridine (α, α' -dipyridyl) in 50 ml ethanol.

Dipping solution Mix equal quantities of solutions I and II immediately before use.

Storage Solution I should be stored in the dark.

Substances 2,2'-Bipyridine
Iron(III) chloride hexahydrate
Ethanol

Reaction

Ascorbic acid, for example, is oxidized to dehydroascorbic acid with reduction of the iron(III) ions. The Fe(II) ions so produced react with 2,2'-bipyridine with formation of a colored complex.

Mobile phase Toluene — chloroform (10 + 10).

Migration distance 5 cm

Running time 10 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in the freshly prepared reagent mixture for 1 s and then dried in a stream of cold air. After a short time α -tocopherol yielded a red chromatogram zone on a colorless background at R_f 35–40. The visual detection limit on the HPTLC layer was 20–25 ng per chromatogram zone.

In situ quantitation: The plate was scanned with visible light ($\lambda = 520$ nm) in the reflectance mode (Fig. 1).

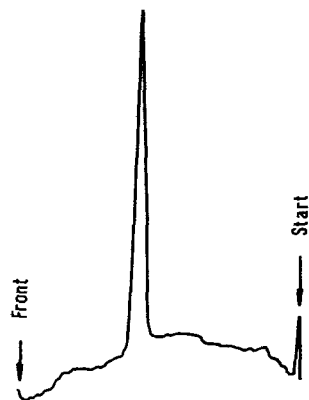


Fig. 1: Reflectance scan of a chromatogram of 200 ng D- α -tocopherol per chromatogram zone.

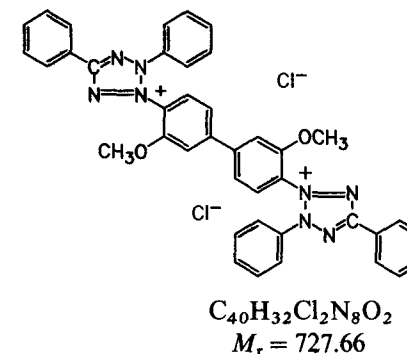
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Blue Tetrazolium Reagent

Reagent for:

- Corticosteroids [1–6]
- Reducing steroids [2]
- Carbohydrates [7]



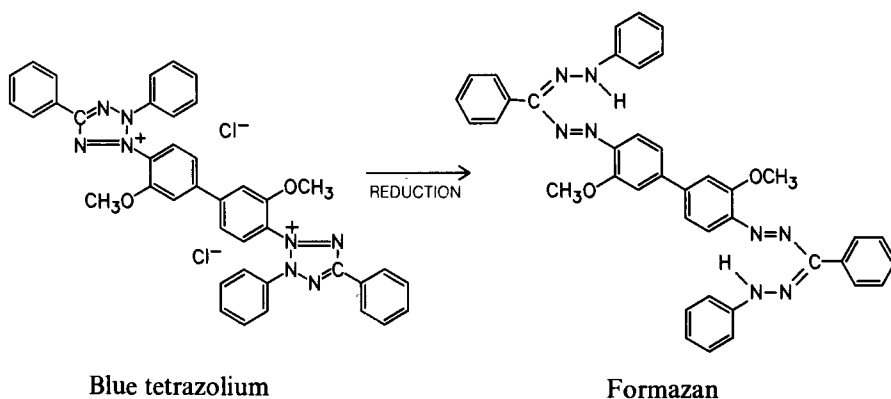
Preparation of Reagent

- Solution I** Dissolve 0.5 g blue tetrazolium (3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-bis(2,5-diphenyl-2H-tetrazolium)-chloride) in 100 ml methanol.
- Solution II** Dissolve 24 g sodium hydroxide carefully in 50 ml water and make up to 100 ml with methanol.
- Dipping solution** Mix equal quantities of solution I and solution II before use.
- Spray solution** Dilute the dipping solution with methanol in a ratio of 1 + 2.
- Storage** Each solution may be stored for a longer period of time in the refrigerator.

Substances Blue tetrazolium
 Sodium hydroxide pellets
 Methanol

Reaction

Blue tetrazolium is transformed into the colored or fluorescent formazan by reducing compounds.



Method

The developed chromatograms are either immersed briefly in the dipping solution or homogeneously sprayed with the spray solution. The color reaction occurs immediately at room temperature or on gentle heating. In general violet-colored zones are formed on a light background. If they are heated to 90°C for 10 to 20 min Δ^4 -3-ketosteroids exhibit rather characteristic deep yellow fluorescence under long-wavelength UV light ($\lambda = 365$ nm).

Note: The reagent can be employed on silica gel, alumina, polyamide and cellulose layers. In the case of the latter it is to be recommended that the solutions be diluted 1 + 3 with methanol. The detection limit is reported to be 0.1 to 0.5 μ g per chromatogram zone [5].

Procedure Tested

Corticosteroids [8]

Method Ascending, one-dimensional, double development in a trough chamber with chamber saturation (5 min intermediate drying in a stream of warm air).

Layer TLC or HPTLC plates Silica gel 60 F₂₅₄ (MERCK).

Mobile phase Chloroform – methanol (93 + 7).

Migration distance TLC: 2 × 12 cm; HPTLC: 2 × 6 cm.

Running time TLC: 2 × 35 min; HPTLC: 2 × 20 min.

Detection and result: The chromatogram was freed from mobile phase and evenly sprayed with reagent solution. After a short time at room temperature violet-colored chromatogram zones appeared for the corticosteroids tetrahydrocortisol (hR_f 10–15), tetrahydrocortisone (hR_f 15–20), prednisolone (hR_f 15–20), hydrocortisone (hR_f 20–25), prednisone (hR_f 30–35), cortisone (hR_f 35–40), corticosterone (hR_f 45–50), cortexolone (REICHSTEIN S., hR_f 50–55), 11-dehydrocorticosterone (hR_f 60–65), 11-desoxycorticosterone (hR_f 75–80).

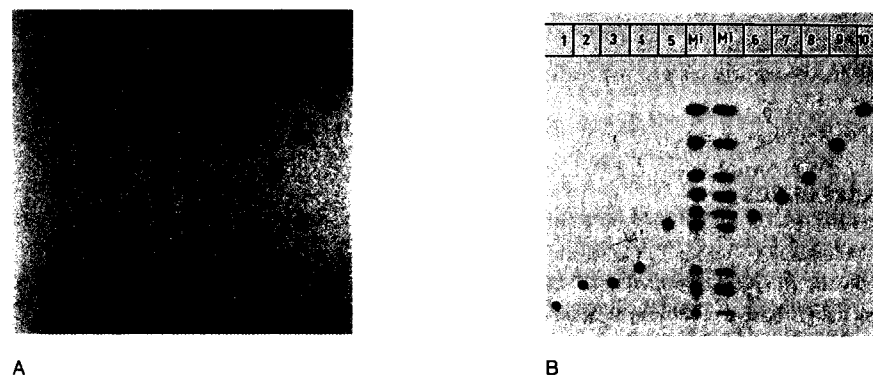


Fig. 1: Separation of the corticosteroids. (A) Detection in UV light ($\lambda = 254$ nm), (B) staining with blue tetrazolium. Tetrahydrocortisol (1), tetrahydrocortisone (2), prednisolone (3), hydrocortisone (4), prednisone (5), cortisone (6), corticosterone (7), cortexolone (8), 11-dehydrocorticosterone (9), 11-desoxycorticosterone (10), mixture (Mi).

Note: In the case of HPTLC plates the detection limit for the visual recognition of the violet ($\lambda_{\max} = 530 \text{ nm}$) colored chromatogram zones was 20 ng per chromatogram zone. With the exception of the two tetrahydrosteroids the corticosteroids could be detected on TLC plates with fluorescent indicators by reason of fluorescence quenching (Fig. 1A). Figure 2 illustrates the absorption scans of the separations illustrated in Figures 1A and 1B.

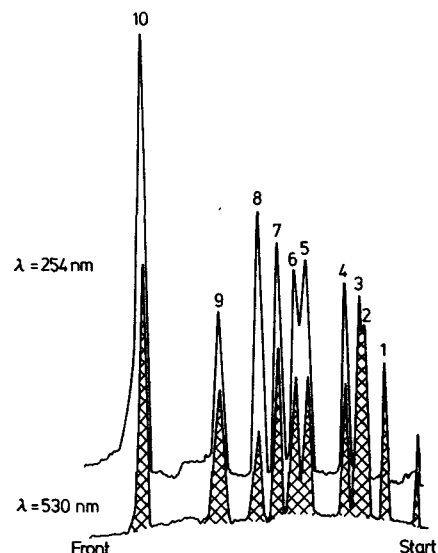


Fig. 2: Absorption scan of the corticosteroid mixture in Figure 1.

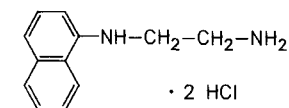
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Bratton-Marshall Reagent

Reagent for:

- Primary aromatic amines [1–3]
e.g. benzodiazepines, aminobenzophenones [3–5]
- Sulfonamides [6–10]
- Urea, carbamate and anilide herbicides [11]
- Folic acid [12]
- Dulcin [13]



• 2 HCl

$\text{C}_{12}\text{H}_{16}\text{Cl}_2\text{N}_2$

$M_r = 259.18$

N-(1-Naphthyl)ethylenediamine dihydrochloride

Preparation of Reagent

Spray solution I Dissolve 1 g sodium nitrite in 20 ml water and make up to 100 ml with a mixture of 17 ml conc. hydrochloric acid and 83 ml ethanol [11].

Spray solution IIa Dissolve 1 g *N*-(1-naphthyl)ethylenediamine dihydrochloride in 10 ml water and make up to 100 ml with ethanol [11].

Spray solution IIb Dissolve 1 g *N*-(1-naphthyl)ethylenediamine dihydrochloride in 50 ml dimethylformamide and 50 ml hydrochloric acid ($c_{\text{HCl}} = 4 \text{ mol/l}$) with warming. If the cooled solution is not clear it should be filtered. A pale violet coloration does not interfere with the reaction [4].

Storage

Spray solutions I and IIa are only stable for a short period of time and, hence, should always be freshly made up [11]. spray solution IIb can be stored for several weeks in the refrigerator [4].

Note: In the case of HPTLC plates the detection limit for the visual recognition of the violet ($\lambda_{\max} = 530 \text{ nm}$) colored chromatogram zones was 20 ng per chromatogram zone. With the exception of the two tetrahydrosteroids the corticosteroids could be detected on TLC plates with fluorescent indicators by reason of fluorescence quenching (Fig. 1A). Figure 2 illustrates the absorption scans of the separations illustrated in Figures 1A and 1B.

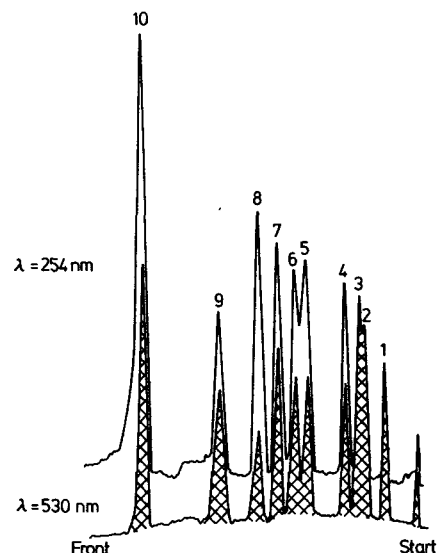


Fig. 2: Absorption scan of the corticosteroid mixture in Figure 1.

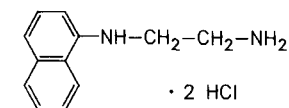
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Bratton-Marshall Reagent

Reagent for:

- Primary aromatic amines [1–3]
e.g. benzodiazepines, aminobenzophenones [3–5]
- Sulfonamides [6–10]
- Urea, carbamate and anilide herbicides [11]
- Folic acid [12]
- Dulcin [13]



• 2 HCl

$\text{C}_{12}\text{H}_{16}\text{Cl}_2\text{N}_2$

$M_r = 259.18$

N-(1-Naphthyl)ethylenediamine dihydrochloride

Preparation of Reagent

Spray solution I Dissolve 1 g sodium nitrite in 20 ml water and make up to 100 ml with a mixture of 17 ml conc. hydrochloric acid and 83 ml ethanol [11].

Spray solution IIa Dissolve 1 g *N*-(1-naphthyl)ethylenediamine dihydrochloride in 10 ml water and make up to 100 ml with ethanol [11].

Spray solution IIb Dissolve 1 g *N*-(1-naphthyl)ethylenediamine dihydrochloride in 50 ml dimethylformamide and 50 ml hydrochloric acid ($c_{\text{HCl}} = 4 \text{ mol/l}$) with warming. If the cooled solution is not clear it should be filtered. A pale violet coloration does not interfere with the reaction [4].

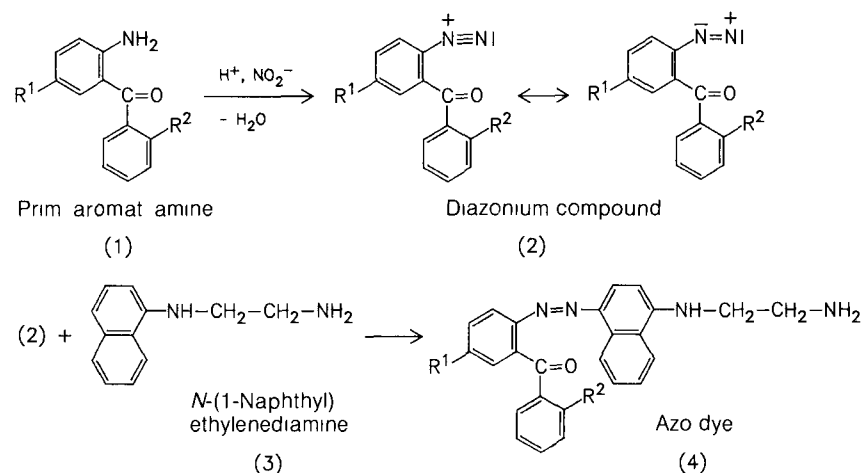
Storage

Spray solutions I and IIa are only stable for a short period of time and, hence, should always be freshly made up [11]. spray solution IIb can be stored for several weeks in the refrigerator [4].

Substances
 Sodium nitrite
N-(1-Naphthyl)ethylenediamine
 dihydrochloride
 Hydrochloric acid 32%
 Ethanol
N,N-Dimethylformamide

Reaction

Primary aromatic amines are first diazotized and then coupled to yield azo dyestuffs [4].



Method

The chromatograms are freed from mobile phase and then sprayed with spray solution I until the layer begins to be transparent, then dried in a stream of cold air for 10 min and finally sprayed again to the start of transparency this time with spray solution IIa or IIb and dried in a stream of warm air.

The pink to violet-colored chromatogram zones on a colorless background usually appear immediately.

Note: Note that the diazotization of primary aromatic amines can also be achieved by placing the chromatogram for 3–5 min in a twin-trough chamber containing nitrous fumes (fume cupboard!). The fumes are produced in the empty trough of the chamber by addition of 25% hydrochloric acid to a 20% sodium nitrite solution [2, 4]. *N*-(1-Naphthyl)ethylenediamine can be replaced in the reagent by α - or β -naphthol [10, 14], but this reduces the sensitivity of detection [2]. Spray solutions IIa and IIb can also be used as dipping solutions.

A range of benzodiazepines which do not contain free aromatic amino groups are only able to react with BRATTON-MARSHALL reagent after in situ hydrolysis to the corresponding aminobenzophenones [4]. In such cases the chromatograms are sprayed with 25% hydrochloric acid after being freed from mobile phase and then heated to 110°C for 10 min before diazotization [4]. Some benzodiazepines form secondary amines on hydrolysis which can be photolytically dealkylated to the corresponding primary amines by irradiation with UV light [4].

Urea, carbamate and anilide herbicides, which are reported only to be detectable on layers containing fluorescence indicators [11], also have to be hydrolyzed, for example by spraying the dried chromatogram with a mixture of 60 ml conc. hydrochloric acid and 50 ml ethanol and then heating (10 min 180°C, covering the chromatogram with a glass plate) before reacting with BRATTON-MARSHALL reagent.

Folic acid is detected by irradiating the chromatogram with broad-spectrum UV light for 30 min before reaction with BRATTON-MARSHALL reagent (detection limit: 200 ng) [12].

The detection limits for benzodiazepines, aminobenzophenones and sulfonamides lie in the lower nanogram range.

The reagent can be employed on silica gel, cellulose and RP layers.

Procedure Tested

Hydrolysis Products of Benzodiazepines [15]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK). Before sample application the plate was prewashed by developing once with chloroform – methanol (50 + 50) and then dried at 110°C for 30 min.

Mobile phase	Benzene.
Migration distance	6 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase (10 min in a stream of warm air) and placed for 10 min in the empty half of a twin-trough chamber in whose second half nitrous fumes were being generated by the addition of 10 drops 37% hydrochloric acid to 5 ml 20% aqueous sodium nitrite solution. After the nitrous fumes had cleared (3–5 min in air, fume cupboard!) the chromatogram was immersed in solution IIa for 1 s and dried in a stream of cold air.

(2-Amino-5-bromophenyl(pyridin-2-yl)methanone ("ABP", deep violet, $hR_f < 5$), 2-amino-5-nitrobenzophenone ("ANB", pink, hR_f 15–20), 2-amino-5-chloro-

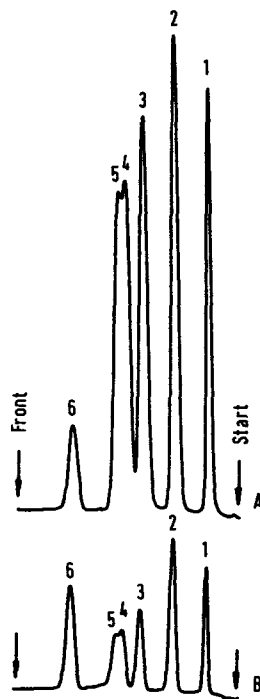


Fig. 1: Reflectance scan (A: scan at $\lambda = 560$ nm, B: scan at $\lambda = 470$ nm) of a chromatogram track of a mixture of 100 ng each of ABP, ANB, ACB, ACFB and ADB and 500 ng MACB per chromatogram zone: ABP (1), ANB (2), ACB (3), ACFB (4), ADB (5), MACB (6).

benzophenone ("ACB", violet, hR_f 30–35), 2-amino-5-chloro-2'-fluorobenzophenone ("ACFB", violet, hR_f 35–40), 2-amino-2',5-dichlorobenzophenone ("ADB", violet, hR_f 40–50) and 5-chloro-2-(methylamino)benzophenone ("MACB", ochre, hR_f 60–65) appeared as colored zones on a white background. The detection limits per chromatogram zone were 5 ng for ABP, ANB, ACB, ACFB and ADB and ca. 50 ng for MACB.

In situ quantitation: The absorption photometric analysis in reflectance was performed at a mean wavelength of $\lambda = 560$ nm ($\lambda_{\max}(\text{ABP}) = 580$ nm, $\lambda_{\max}(\text{ANB}) = 550$ nm, $\lambda_{\max}(\text{ACB, ACFB, ADB}) = 560$ nm, $\lambda_{\max}(\text{MACB}) = 470$ nm, see Fig. 1).

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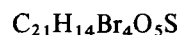
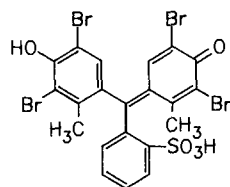
Bromocresol Green — Bromophenol Blue — Potassium Permanganate Reagent

Reagent for:

- Organic acids

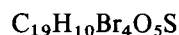
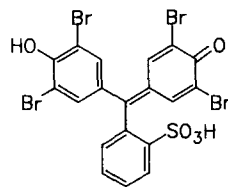


$$M_r = 158.04$$



$$M_r = 698.04$$

Bromocresol green



$$M_r = 669.96$$

Bromophenol blue

Preparation of the Reagent

Solution I	Dissolve 40 mg bromocresol green and 15 mg bromophenol blue in 100 ml ethanol.
Solution II	Dissolve 250 mg potassium permanganate and 500 mg sodium carbonate in 100 ml water.
Dipping solution	Mix solutions I and II to a ratio of 9 + 1 immediately before use.
Storage	Solutions I and II may be stored in the refrigerator for an extended period. The dipping solution must be employed within 5–10 min of preparation.
Substances	Bromocresol green Bromophenol blue Potassium permanganate Sodium carbonate decahydrate Ethanol

Reaction

The detection of acids takes place on the basis of the pH-dependent color change of the two indicators bromocresol green (pH range: 3.8–5.4) and bromophenol blue (pH range: 3.0–4.6) from yellow to blue.

Method

The chromatogram is freed from mobile phase in a stream of warm air or in the drying cupboard (5 min 100°C), immersed for 1 s in freshly prepared dipping solution or sprayed homogeneously with it and then heated to 100°C for 5–10 min. This usually results in the formation of blue-green colored zones on a grey-blue background.

Note: The reagent can be employed on silica gel, kieselguhr, RP and, with lower sensitivity of detection, on cellulose layers. The color differentiation is probably greater on cellulose layers [1]. A dark blue background is produced on polyamide layers.

Procedure Tested

Organic Acids [2]

Method	Ascending, one-dimensional development with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Diisopropyl ether – formic acid – water (90 + 7 + 3).
Migration distance	10 cm
Running time	30 min

Detection and result: The chromatogram was freed from mobile phase in the drying cupboard (5 min 100°C), immersed in the freshly prepared dipping reagent for 1 s and then heated to 100°C for 10 min.

Even before heating all the acids rapidly appeared as blue zones on a yellow-blue background. After heating tartaric acid (hR_f 2–5) and malic acid (hR_f 5–10) retained their color while lactic acid (hR_f 30–35), succinic acid (hR_f 35–40), pimelic acid (hR_f 50), maleic acid (hR_f 55), suberic acid (hR_f 55–60), benzoic acid (hR_f 80–85), stearic acid (hR_f 85–90) and arachidic acid (hR_f 85–90) appeared as pale yellow zones on a blue-yellow background (Fig. 1). The detection limits lay at 1 to 2 μg substance per chromatogram zone.

In situ quantitation: This reagent was not suitable for direct, precise photometric quantitation.

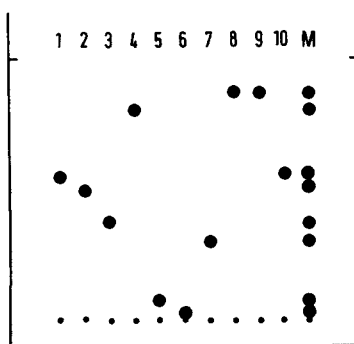


Fig. 1: Schematic representation of the chromatographic separation of carboxylic acids. Maleic acid (1), pimelic acid (2), succinic acid (3), benzoic acid (4), malic acid (5), tartaric acid (6), lactic acid (7), stearic acid (8), arachidic acid (9), suberic acid (10), mixture (M).

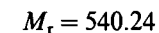
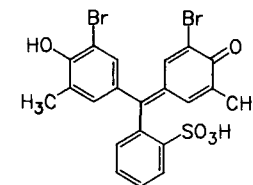
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Bromocresol Purple Reagent

Reagent for:

- Organic acids [1–4]
- Halogen anions [5]
- Phenols [6]
- 5-Aminodibenzo(a,d)cycloheptane derivatives [7]



Preparation of the Reagent

Dipping solution I *Organic acids:* Dissolve 40 mg bromocresol purple in 100 ml 50% ethanol and adjust to pH = 10.0 (glass electrode) with caustic soda solution ($c = 0.1 \text{ mol/l}$) [1].

Dipping solution II *Halogen anions:* Dissolve 100 mg bromocresol purple in 100 ml ethanol and add a few drops of 10% ammonia solution until the color changes.

Storage The bromocresol solution keeps well in the refrigerator before basification.

Substances Bromocresol purple
Sodium hydroxide solution (0.1 mol/l)
Ammonia solution (25%)
Ethanol

Reaction

The pH indicator bromocresol purple changes in color from yellow to purple in the pH range 5.2–6.8.

Method

The chromatogram is freed from mobile phase in a stream of warm air or in the drying cupboard (10 min 100°C) and after cooling dipped for 1 s in dipping solution I or II or sprayed homogeneously with it. Then it is heated to 100°C for 10 min.

Organic acids yield lemon-yellow zones on a blue background [1]. Halide ions migrate as ammonium salts in ammoniacal mobile phases and are also colored yellow. The colors fade rapidly in the air. This can be delayed for some days by covering the chromatogram with a glass plate.

Note: The background color depends on the pH of the layer, it is, therefore, affected by the efficiency of removal of acidic mobile phase components before staining.

Dicarboxylic acids react more sensitively than do monocarboxylic acids. Fatty acids and amino acids cannot be detected.

The reagent can be employed on silica gel, kieselguhr and Si 50 000 layers (also when they are impregnated with polyethylene glycol [1]) and on cellulose layers.

Procedure Tested

Multibasic Carboxylic Acids [8]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	1. TLC plates Silica gel 60 (MERCK). 2. TLC plates Silica gel 60 (MERCK) impregnated by dipping once in a 10% solution of polyethylene glycol 1000 in methanol.
Mobile phase	Diisopropyl ether — formic acid — water (90 + 7 + 3).
Migration distance	10 cm
Running time	30 min (silica gel layer) 50 min (impregnated layer)

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air or in the drying cupboard (10 min 100°C), immersed in dipping

solution I for 1 s and then heated on a hotplate to 100°C for 15 min. Citric acid (hR_f 0–5), lactic acid (hR_f 30), phthalic acid (hR_f 40–45), sebacinic acid (hR_f 60–65) and salicylic acid (hR_f 80–85) yielded lemon-yellow chromatogram zones on a yellow background on silica gel layers (Fig. 1). Figure 2 illustrates a similar separation of other dicarboxylic acids.

In situ quantitation: The reagent was not suitable for an exact quantitative determination of acids because the background structure did not allow sensitive reproducible scanning.

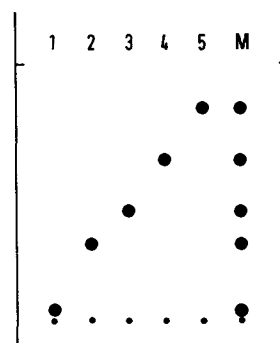


Fig. 1

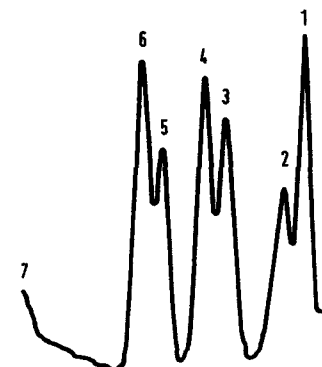


Fig. 2

Fig. 1: Separation of carboxylic acids (schematic representation). Citric acid (1), lactic acid (2), phthalic acid (3), sebacinic acid (4), salicylic acid (5), mixture (M).

Fig. 2: Separation of dicarboxylic acids (reflectance scan). Start + tartaric acid (1), malic acid (2), phthalic acid (3), succinic acid (4), adipic acid (5), fumaric acid (6), front (7).

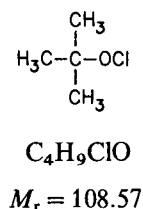
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tert-Butyl Hypochlorite Reagent

Reagent for:

- Fatty acids, triglycerides, amino acids, sugars, steroids [1]
- Peptides, nucleotides [2]
- Vitamin B₁ [3]
- Alkaloids [5]
- Carbamazepine [4]



Preparation of the Reagent

Dipping solution I Dissolve 1 ml *tert*-butyl hypochlorite in 100 ml carbon tetrachloride or cyclohexane.

Dipping solution II Mix chloroform, paraffin oil and triethanolamine in the ratio of 6 + 1 + 1.

Storage Dipping solution I may be stored in the refrigerator for a few days. *tert*-Butyl hypochlorite should be stored cool in the dark under an atmosphere of nitrogen.
Caution: *tert*-Butyl hypochlorite is very reactive, e.g. it reacts violently with rubber seals particularly under the influence of light.

Substances *tert*-Butyl hypochlorite
Carbon tetrachloride
Paraffin viscous
Chloroform
Triethanolamine

Reaction

The reaction mechanism has not been elucidated.

Method

The developed chromatogram is freed from mobile phase in a stream of warm air and then either immersed in dipping solution I for 1 s or sprayed homogeneously with it or exposed to its vapors for 15 min in a twin-trough chamber in whose second chamber 5 ml of dipping solution I has been placed ca. 10 min previously. The chromatograms are then immersed in dipping solution II for 1 s and dried in a stream of hot air. If derivatization is carried out by dipping, the chromatograms should be dried for ca. 1 min in a stream of hot air and allowed to cool to room temperature before the second treatment.

There then appear, in long-wavelength UV light ($\lambda = 365$ nm), yellow to violet fluorescent zones on a dark background; these can be quantitatively analyzed.

Note: The reagent can be employed on silica gel and cellulose layers. When derivatization is carried out from the vapor phase the detection limit for morphine is 10 ng and that for papaverine 1 ng per chromatogram zone [5]. In some cases it has been recommended that ammonium sulfate be added to the layer with subsequent heating to 150–180°C [1] after derivatization. It is also possible to spray afterwards with an aqueous solution of potassium iodide (1%) and starch (1%) [2].

Procedure Tested

Vitamin B₁ [3]

Method	Ascending, one-dimensional development in a twin-trough chamber with chamber saturation with the layer being pre-conditioned in the solvent-free half of the trough for 15 min after application of the sample.
Layer	HPTLC plates Silica gel 60 (MERCK), which had been pre-washed by single development of the plate with chloroform – methanol (50 + 50) and then dried at 110°C for 30 min.
Mobile phase	Methanol – ammonia solution (25%) – glacial acetic acid (8 + 1 + 1).
Migration distance	6 cm
Running time	ca. 20 min

Detection and result: The chromatogram was dried in a current of warm air and either immersed in reagent solution I for 1 s or placed for 15 min in a twin-trough chamber in whose second trough 5 ml of dipping solution I had been placed ca. 10 min previously. If the chromatogram was derivatized by dipping it had to be dried for ca. 1 min in a stream of hot air and allowed to cool to room temperature.

The chromatogram was then immersed for 1 s in reagent solution II to increase sensitivity and stabilize the fluorescence and then dried in a stream of hot air.

Thiamine (hR_f 40–45) yielded a yellow fluorescent zone on a dark background under long-wavelength UV light ($\lambda = 365$ nm).

Note: Vapor-phase derivatization was appreciably more homogeneous than that produced by dipping and simultaneously increased both the precision and the sensitivity of the method.

In situ quantitation: The fluorimetric analysis was performed in long-wavelength UV light ($\lambda_{exc} = 365$ nm, $\lambda_{fl} > 430$ nm). The detection limit for thiamine was less than 3 ng per chromatogram zone.

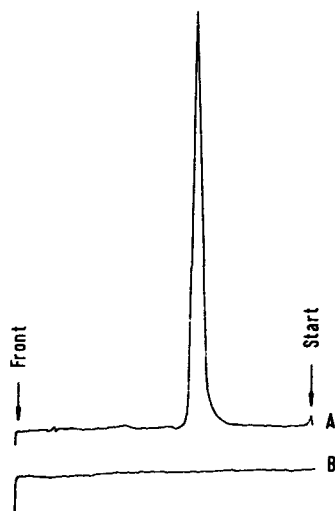


Fig. 1: Fluorescence scan of 30 ng thiamine after vapor-phase derivatization (A) and an accompanying blank track (B).

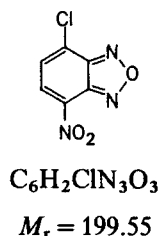
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7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole Reagent (NBD-Chloride Reagent)

Reagent for:

- Prim. and sec. aliphatic amines [1–3]
- Amino acids and peptides [1, 4, 5]
- Sulfonamides [6]
- Alkaloids [7]
- Phenols [7]



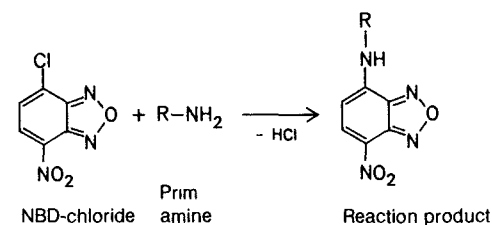
Preparation of Reagent

- Dipping solution I** Dissolve 10 g sodium acetate in 20 ml water and dilute with 40 ml methanol.
- Dipping solution II** Dissolve 0.1 g NBD-chloride (7-chloro-4-nitrobenzofurazan, 7-chloro-4-nitro-2,1,3-benzoxadiazole) in 50 ml ethanol.
- Spray solution I** Dissolve 2 g sodium carbonate or 5 g sodium hydroxide in 100 ml water [6, 7].
- Spray solution II** Dissolve 0.1–0.5 g NBD-chloride in 100 ml ethanol [5], methanol [6] or acetonitrile [7].
- Storage** All solutions may be stored for an extended period of time.
- Substances** 7-Chloro-4-nitrobenzofurazan
Sodium acetate trihydrate
Sodium carbonate

Sodium hydroxide pellets
Methanol
Ethanol

Reaction

NBD-chloride reacts with nucleophilic compounds (amines, mercaptans etc.) to yield the corresponding 7-substituted 4-nitrobenzofurazan derivatives.



Method

The dried chromatograms are immersed in dipping solution I for 1 s, dried in a stream of warm air, dipped immediately after cooling in dipping solution II (1 s) and then heated to 100 °C for 2–3 min. Alternatively the chromatogram can be sprayed with the appropriate spray solutions.

Under long-wavelength UV light ($\lambda = 365$ nm) the chromatogram zones fluoresce yellow to yellow-green, they are sometimes detectable in daylight as colored zones, too.

Note: The reagent can be employed for qualitative and quantitative analysis on silica gel and RP layers. Ammonia, amine and acid-containing mobile phases should be completely removed beforehand. Amino phases cannot be employed. The NBD-chloride reagent is not as sensitive as the DOOB reagent (qv.) on RP phases.

Primary and secondary amines and phenols generally produce yellow to reddish-orange zones (serotonin violet) on a pale yellow background, under long-wave-

length UV light ($\lambda = 365$ nm) the zones fluoresce yellowish green (serotonin appears as a dark zone). The plate background also fluoresces but appreciably less. Noradrenaline, 5-hydroxytryptophan and dopamine yield red to blue-violet zones on a yellow background, they do not fluoresce*. Sulfonamides and alkaloids produce greenish-yellow, olive brown or violet colors. Peptides and amino acids (except tryptophan) yield pale yellow fluorescent zones; tryptophan and proline produce orange-red zones. The detection limits are 100–800 ng substance per chromatogram zone, sometimes appreciably less (cf. "Procedure Tested" [8]).

Although the reagent itself is not fluorescent an excess of NBD-chloride can interfere in quantitative analysis. In such cases it should be checked whether prechromatographic derivatization produces better results [3, 4]. The reaction products can then be separated on polyamide layers.

Procedure Tested

Proline, Hydroxyproline [8]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK), which had been developed once with chloroform – methanol (50 + 50) and dried at 110°C for 30 min before applying the samples.
Mobile phase	Chloroform – methanol – ammonia solution (25%) (100 + 90 + 20).
Migration distance	7 cm
Running time	40 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, then immersed in dipping solution I for 1 s, dried for 90 s in a stream of warm air and after cooling immersed in dipping solution II for 1 s. After drying briefly in a stream of warm air it was heated to 110°C for 2 min and treated with hydrochloric acid vapor for 15 min in a twin-trough chamber (37% hydrochloric acid in the vacant trough).

* Jork, H.: private communication, Universität des Saarlandes, Fachbereich 14 "Pharmazie und Biologische Chemie", D-6600 Saarbrücken 1988.

Hydroxyproline (*hR_f* 20–25) and proline (*hR_f* 25–30) yielded orange-colored chromatogram zones on a pale yellow background, under long-wavelength UV light ($\lambda = 365$ nm) they fluoresced yellow on a blue background. (Detection limits: ca. 5 ng substance per chromatogram zone).

The chromatogram was then immersed for 1 s in a mixture of liquid paraffin and *n*-hexane (1 + 2) to stabilize and enhance the fluorescence (by a factor of ca. 1.5). The detection limits were then ca. 1 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric measurement in reflectance was performed at $\lambda = 490$ nm, the fluorimetric analysis at $\lambda_{exc} = 436$ nm and $\lambda_{fl} > 560$ nm (Fig. 1).

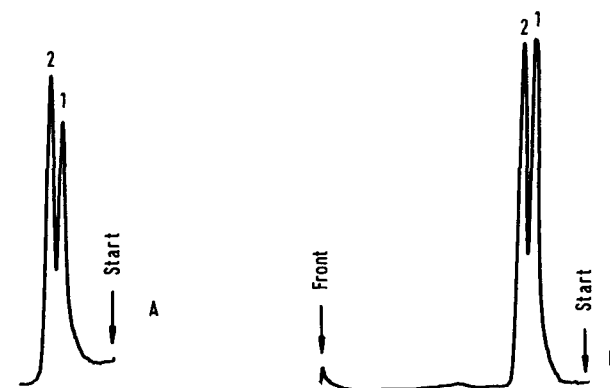


Fig. 1: Reflectance scan (A) and fluorescence scan (B) of a chromatogram track with 50 ng of each amino acid per chromatogram zone: hydroxyproline (1), proline (2).

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Copper(II) Acetate — Phosphoric Acid Reagent

Reagent for:

- Prostaglandins [1–3]
 - β -Sitosterol [4]
 - Polar lipids [5]
 - Phospholipids [5–17]
 - Glycolipids [9]
 - Esters of fatty acids [18, 19]
- | | |
|---------------------------------------------------------------|-------------------------------|
| $(\text{CH}_3\text{COO})_2\text{Cu} \cdot \text{H}_2\text{O}$ | H_3PO_4 |
| $M_r = 199.65$ | $M_r = 98.00$ |
| Copper(II) acetate monohydrate | <i>ortho</i> -Phosphoric acid |

Preparation of the Reagent

- Dipping solution** Dissolve 3 g copper(II) acetate in 100 ml 8–15% aqueous phosphoric acid.
- Storage** The dipping solution can be stored for long periods of time.
- Substances** *ortho*-Phosphoric acid (85%)
Copper(II) acetate monohydrate

Reaction

The reaction mechanism has not yet been elucidated.

Method

The chromatograms are dried in a stream of warm air, immersed in the reagent solution for 2 s and then heated to 120–125°C for 10–15 min. β -Sitosterol appears as a grey-blue zone on a colorless background [4] and prostaglandins yield variously colored zones [2], which absorb long-wavelength UV light ($\lambda = 365$ nm). The dipping solution can also be employed as a spray solution [11].

Note: Phospholipids and glycolipids appear after 6–25 min [5–13] and esters of fatty acids after heating for 2 h [18] at 180°C (charring!) as grey-black zones on an almost colorless background. According to TOUCHSTONE [7] in the case of phospholipids it is only the unsaturated ones that react, while the saturated ones only react with the copper(II) sulfate — phosphoric acid reagent. The reagent may be applied to silica gel layers.

Procedure Tested

Prostaglandin E₁ [1]

- Method** Horizontal, one-dimensional development in a linear chamber (CAMAG).
- Layer** HPTLC plates Silica gel 60 (MERCK).
The layer was prewashed twice before application of the samples by developing first with cyclohexane and then, after drying for 30 min at 120°C, with methanol; it was finally activated for 30 min at 120°C.
- Mobile phase** Ethyl acetate — formic acid (80 + 1).
- Migration distance** ca. 5 cm
- Running time** 10–15 min

Detection and result: The chromatogram was dried in a stream of warm air, immersed in the reagent solution for 2 s and then heated to 120°C for 15 min. Prostaglandin E₁ appeared as a yellow-brown zone (R_f 25–30) on a colorless background.

In situ quantitation: Quantitation by reflectance had to be performed as soon as possible, since the color intensity of a zone decreased ca. 40% within a day. Detection wavelength: $\lambda = 345$ nm; detection limit: 2.5 ng per chromatogram zone (Fig. 1).

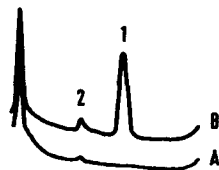


Fig. 1: Reflectance curve of a blank track (A) and a chromatogram (B) with 80 ng prostaglandin E_1 (1), β -front (2).

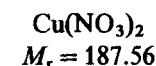
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Copper(II) Nitrate Reagent

Reagent for:

- Stabilization of “ninhydrin” spots [1–3]



Preparation of the Reagent

Dipping solution Mix 1 ml saturated aqueous copper(II) nitrate solution with 0.2 ml 10% nitric acid and make up to 100 ml with 95% ethanol [1].

Storage The reagent can be kept for several days.

Substances Copper(II) nitrate trihydrate
Nitric acid (65%)
Ethanol
Ammonia solution (25%)

Reaction

The blue-violet stain which forms on thin-layer chromatograms when amino acids are stained with ninhydrin is only stable for a short time. It rapidly begins to fade even on cellulose layers. The stability can be appreciably enhanced by complex formation with metal ions [3].

Method

The chromatograms stained with ninhydrin are immersed in the reagent solution for 1 s or sprayed evenly with it and then placed in the free half of a twin-trough chamber containing 25% ammonia solution. Apart from proline and hydroxyproline, which yield yellow copper complexes, all the amino acids yield reddish-colored chromatogram zones [3].

Note: The copper in the reagent may be replaced by other metal ions. Table 1 [3] lists the colors obtainable with some cations.

Table 1: Colors of the metal cation complexes

Amino acid	Zn ²⁺	Cd ²⁺	Co ²⁺	Cu ²⁺
Proline	yellow	yellow	brown	yellow ochre
Hydroxyproline	grey	yellow	light brown	
Alanine	brick red	wine red	brown	salmon
Glycine	brick red	wine red	brown	salmon
Serine	brick red	wine red	brown	salmon
Threonine	brick red	wine red	brown	salmon
Phenylalanine	red	red ochre	brown	red ochre
Tryptophan	red	orange	brown	red ochre
Other amino acids	red	wine red	brown	salmon

The copper complex is very stable at neutral pH, but it fades very rapidly in the presence of hydrogen ions. Other complex formers such as tartaric acid or citric acid and thiourea interfere with the reaction and, therefore, should not be included in mobile phases used for the separation of amino acids [3].

When staining with ninhydrin the appearance of colors of various hues on TLC layers with and without fluorescence indicators is probably a result of complex formation between the "ninhydrin zones" and the cations of the inorganic fluorescence indicators.

Procedure Tested

Valine, Phenylalanine, Leucine, Isoleucine [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation
Layer	HPTLC plates Cellulose (MERCK) Before sample application the layers were prewashed by developing with chloroform — methanol (50 + 50) and then dried at 110 °C for 30 min.

Mobile phase 2-Propanol — 1-butanol — trisodium citrate solution ($c = 0.2$ mol/l in water) (50 + 30 + 20).

Migration distance 9 cm

Running time 120 min

Detection and result: After staining with ninhydrin the chromatogram was cooled to room temperature, immersed in the reagent solution for 1 s and after drying in the air it was then placed for 15 min in the empty half of a twin-trough chamber containing 25% ammonia solution.

Before treatment with ammonia valine (hR_f 45–50), isoleucine (hR_f 65–70) and leucine (hR_f 70–75) produced red chromatogram zones and phenylalanine (hR_f 55–60) violet chromatogram zones on a pale pink background. After treatment with ammonia all four amino acids appeared as violet chromatogram zones on a flesh-colored background; these zones were stable over an extended period.

In situ quantitation: The absorption-photometric quantitation was carried out in reflectance at $\lambda = 540$ nm. The detection limit was 5 ng substance per chromatogram zone.

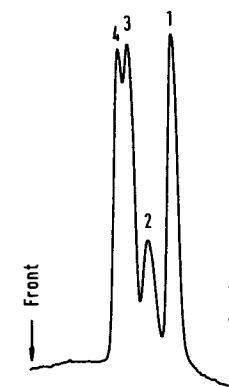


Fig. 1: Reflectance scan of a chromatogram track with ca. 100 ng amino acid per chromatogram zone. Valine (1), phenylalanine (2), isoleucine (3), leucine (4).

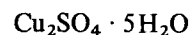
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Copper(II) Sulfate Reagent

Reagent for:

- Di-, tri- and polycarboxylic acids [1]
- Thioglycolic and dithioglycolic acids [2]
- Diuretics [3]
- Antithyroid pharmaceuticals [4]
e.g. N-aryl-N'-benzenesulfonylthiocarbamides
- N-Arylthiosemicarbazides [5]
- Sterols and their esters [6]
e.g. cholesterol and cholesteryl esters
- Metal chelates [7–9]



$$M_r = 249.68$$

Preparation of Reagent

Dipping solution Dissolve 1.5 g copper(II) sulfate pentahydrate in a few milliliters of water and make up to 100 ml with methanol.

Storage The solution should always be freshly made up.

Substances Copper(II) sulfate pentahydrate
Methanol

Reaction

The substances listed above form colored copper complexes.

Method

The chromatograms are freed from mobile phase, immersed in the reagent solution for 10 s or homogeneously sprayed with it and then dried in a stream of warm air. Di- and tricarboxylic acids are reported to appear rapidly as blue zones on an evenly pale blue background [1]. On layers containing fluorescence indicators they can usually also be recognized under short-wavelength UV light ($\lambda = 254$ nm) as dark spots on a green fluorescent background [1]; thioglycolic and dithioglycolic acids yield green-colored zones [2] and N-aryl-N'-benzenesulfonylthiocarbamides yield yellow to violet-colored zones [4].

For the determination of sterols and their esters the chromatogram is immersed in a 10% aqueous copper(II) sulfate solution and then heated to 105°C for 30 min. In this case green-yellow fluorescent chromatogram zones are visible in long-wavelength UV light ($\lambda = 365$ nm) [6].

Note: Silica gel, kieselguhr and polyamide layers can be used as stationary phases. Not all acids are stained on RP layers. Amino layers yield a pale blue background. The detection limits are in the μg range for carboxylic acids [1], thioglycolic and dithioglycolic acids [2] and for antithyroid pharmaceuticals [4]; they are about 5 ng per chromatogram zone for sterols and sterol esters [6].

Table 1: List of carboxylic acids that can be detected with copper(II) sulfate reagent [1]

Acid	Color	Fluorescence quenching ($\lambda = 254$ nm)	Sensitivity ($\mu\text{g}/\text{zone}$)	
			TLC	HPTLC
Malonic acid	pale blue	+	20	
Succinic acid	blue	—	5	
Methyl-succinic acid	blue	—	10	
Adipic acid	blue	—	15	5
Pimelic acid	blue	—	5	
Suberic acid	blue	—	5	
Sebacic acid	blue	—	5	
Fumaric acid	pale blue	+	10	2.5
Maleic acid	blue	+	10	
Aconitic acid	blue	+	10	
Phthalic acid	pale blue	+	30	20
Terephthalic acid	blue	+	15	
α -Ketoglutaric acid	pale blue	+	25	

A mixture of 10% aqueous copper(II) sulfate solution and 25% ammonia solution (100 + 15) is recommended as spray solution for the detection of diuretics.

Procedure Tested

Organic Acids [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC aluminium sheets, TLC plates. Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Diisopropyl ether – formic acid – water (90 + 7 + 3).
Migration distance	10 cm
Running time	40 min

Detection and result: The TLC plate was dried in the air for 30 min and heated to 110 °C for 10 min in order to remove the formic acid from the mobile phase, before immersing the chromatogram in the reagent solution for 10 s.

Tartaric acid (hR_f 5), malic acid (hR_f 15), maleic acid (hR_f 35), lactic acid (hR_f 40, adipic acid (hR_f 45) and fumaric acid (hR_f 55) yielded blue zones on a weakly bluish background; these were still to be recognized unchanged after several weeks.

In situ quantitation: The quantitation (Fig. 1, succinic acid) was performed in reflectance with visible light ($\lambda = 690$ nm).

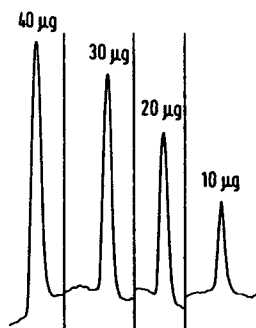


Fig. 1: Absorption scans of a range of dilutions of succinic acid [1].

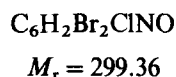
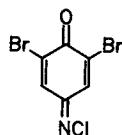
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2,6-Dibromoquinone-4-chloroimide Reagent (Gibbs' Reagent)

Reagent for:

- Phenols [1 – 3, 5]
e.g. vitamin B₆ [1, 3]
- Coumarins [15]
- Thiol and thione compounds [4, 5]
- Antioxidants [6]
- Prim. and sec. aliphatic amines [7]
- Prim., sec. and tert. aromatic amines [7]
- Carbazoles and aromatic hydrocarbons [7]
- Indoles and other N-containing heterocyclics [7]
- 2,4-Pentanedione [7]
- Pesticides [8, 9, 13]
- Antiepileptics [10, 11]
- Barbiturates [10]



Storage

The solutions may be stored in the refrigerator for ca. 2 weeks [3].

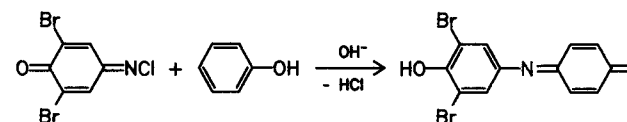
Care: 2,6-dibromoquinone-4-chloroimide can decompose explosively [9]; so only small amounts should be stored in the refrigerator!

Substances

2,6-Dibromoquinone-4-chloroimide
Sodium hydrogen carbonate
Dimethyl sulfoxide (DMSO)
Chloroform
Methanol
Ethanol

Reaction

2,6-Dibromoquinone-4-chloroimide, in whose stead 2,6-dichloroquinone-4-chloroimide (q.v.) may also be employed, reacts primarily with phenols and anilines which are not substituted in the *p*-position.



Method

The chromatogram is dried in a stream of cold air, heated to 110°C for 10 min, cooled and immersed in the dipping solution for 5 s or sprayed with spray solution and finally heated to 110°C for 2 min.

Note: If the spray solution or a nonbasic dipping solution [2] is employed for detection then it is advisable to spray again with a 10% aqueous sodium carbonate solution. The necessary basicity can also often be achieved by placing the treated chromatogram in a twin-trough chamber whose second trough contains 5 ml ammonia solution (25%).

In general no warming is necessary to produce the colored zones: pyridoxine is colored intense blue, pyridoxamine violet and pyridoxal dark blue [1, 3]. Phenols

Preparation of the Reagent

Dipping solution Dissolve 0.1 g 2,6-dibromoquinone-4-chloroimide in 10 ml dimethyl sulfoxide saturated with sodium hydrogen carbonate. Then make up to 100 ml with chloroform.

Spray solution Dissolve 0.4 to 1 g 2,6-dibromoquinone-4-chloroimide in 100 ml methanol or ethanol and filter if necessary.

that are substituted in the *p*-position exhibit characteristic color differences. Thiol compounds and substances containing sulfhydryl groups yield yellow zones, while thiones are colored red. Thiourea appears as a brown zone on a light background [4].

In some cases, e.g. the detection of antioxidants [6], the plate is heated to 105 °C for 5 min after being sprayed and the still hot plate placed immediately in an ammonia-vapor chamber. The blue color of the tryptamine derivatives is also stabilized by spraying afterwards with a 5% methanolic ammonia solution [12].

The sensitivity of detection is usually between 0.2 and 0.5 µg per chromatogram zone. This is also true for pesticides based on organophosphorus acids [9].

The reagent can be employed on silica gel, cellulose and polyamide layers.

Procedure Tested

Antiepileptics, Barbiturates [10]

Method	Ascending, one-dimensional development in a trough chamber. After sample application the HPTLC plates were equilibrated in a conditioning chamber at 42% relative humidity for 30 min and then developed immediately.
Layer	HPTLC plates Silica gel 60 (MERCK) which were prewashed by developing three times in chloroform – methanol (50 + 50) and drying at 110 °C for 30 min.
Mobile phase	Chloroform – acetone (80 + 15).
Migration distance	7 cm
Running time	20 min

Detection and result: The chromatogram was freed from mobile phase (first dried for 5 min in a stream of cold air, then heated for 10 min at 110 °C and allowed to cool), immersed for 5 s in the reagent solution and finally heated for 2 min at 110 °C. Grey to grey-blue zones were formed on a light background. The following *hR_f* values were obtained: primidone (*hR_f* 10–15); carbamazepine (*hR_f* 40–45); phenytoin (*hR_f* 50–55); phenobarbital (*hR_f* 60); ethosuximide (*hR_f* 75); hexobarbital (*hR_f* 90–95).

In situ quantitation: Reflectance measurements were carried out at a wavelength of $\lambda = 429$ nm. The detection limit lay at 50 to 200 ng per chromatogram zone (Fig. 1).

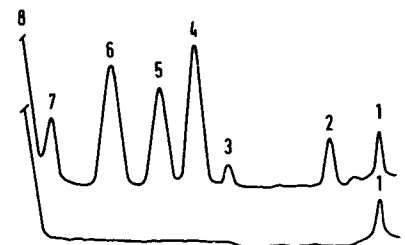


Fig. 1: Reflectance scan of a blank (A) and of a mixture of antiepileptics with 500 ng substance per chromatogram zone (B). Start (1), primidone (2), carbamazepine (3), phenytoin (4), phenobarbital (5), ethosuximide (6), hexobarbital (7) and solvent front (8).

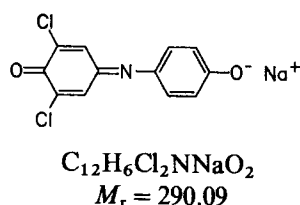
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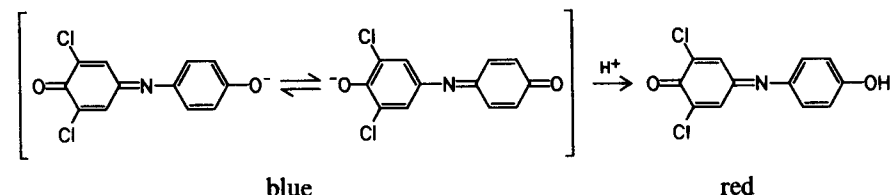
2,6-Dichlorophenolindophenol Reagent (Tillmans' Reagent)

Reagent for:

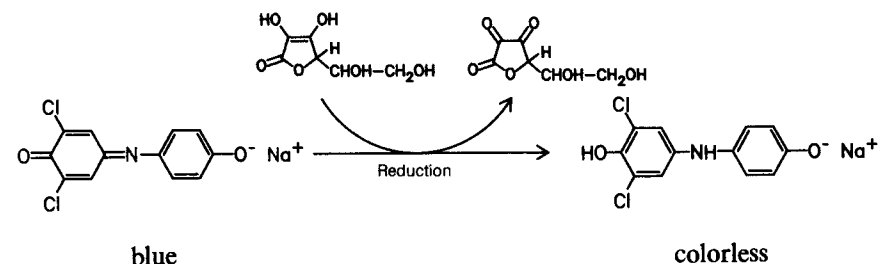
- Organic acids [1, 2]
- Reductones
e.g. ascorbic acid (vitamin C) [2, 3]



Organic acids:



Reductones:



Preparation of the Reagent

Dipping solution	Dissolve 40 mg 2,6-dichlorophenolindophenol sodium in 100 ml ethanol.
Spray solution	Dissolve 100 mg 2,6-dichlorophenolindophenol sodium in 100 ml ethanol [1, 2].
Storage	The reagent solutions may be stored in the refrigerator for several weeks.
Substances	2,6-Dichlorophenolindophenol sodium salt Ethanol

Reaction

Organic acids convert the blue mesomerically stabilized phenolate anion to the red undissociated acid. Reductones (e.g. ascorbic acid) reduce the reagent to a colorless salt.

Method

The chromatograms are freed from mobile phase, immersed in the dipping solution for 1 s or sprayed evenly with the spray solution and heated to 100°C for 5 min. Carboxylic acids yield red-orange zones and reductones colorless zones on a blue-violet background.

Note: Acids contained in the mobile phase must be completely removed otherwise pale violet zones will be produced instead of the red-orange zones on a violet background. If heating is prolonged the pink color of keto acids changes to white [1]. The colored zones of the carboxylic acids appear sharper if the chromatograms are exposed to an atmosphere of ammonia for a few seconds after heating [1]. Ascorbic and dehydroascorbic acids can also be detected fluorimetrically if the chromatogram is immersed after heating in liquid paraffin — *n*-hexane (1 + 2). In long-wavelength UV light ($\lambda = 365$ nm) they appear as green fluorescent zones on a dark background [3].

The reagent can be employed on silica gel, kieselguhr, Si 50 000, cellulose and polyamide layers.

Procedure Tested

Organic Acids [4]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 (MERCK).
Mobile phase	Diisopropyl ether – formic acid – water (90 + 7 + 3).
Migration distance	10 cm
Running time	30 min

Detection and result: The chromatogram was freed from mobile phase, immersed for 1 s in the dipping reagent and then heated on a hotplate at 100°C for 5 min.

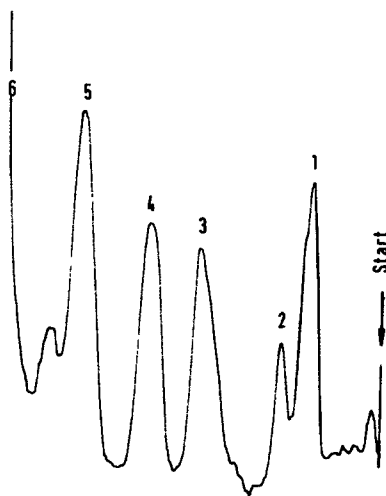


Fig. 1: Absorption scan of a chromatogram with 10 µg (!) per chromatogram zone of the carboxylic acids tartaric acid (1), malic acid (2), lactic acid (3), succinic acid (4), fumaric acid (5), stearic acid + front (6).

The carboxylic acids tartaric acid (hR_f 15–20), malic acid (hR_f 25–30), lactic acid (hR_f 45–50), succinic acid (hR_f 60–65), fumaric acid (hR_f 80) and stearic acid (hR_f 95–100) yielded orange-red zones on a violet background.

In situ quantitation: The photometric evaluation was carried out at $\lambda = 600$ nm. The detection limits of the acids were at 200–500 ng per chromatogram zone (Fig. 1).

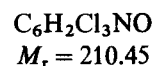
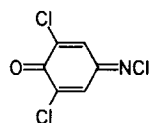
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2,6-Dichloroquinone-4-chloroimide Reagent

Reagent for:

- Phenols [1–3, 6]
e.g. vitamin B₆
- Antioxidants [2–4]
- Prim. and sec. aliphatic amines [5]
- Prim., sec. and tert. aromatic amines [5]
- Carbazoles and aromatic hydrocarbons [5]
- Indoles and other N-containing heterocyclics [5]
- 2,4-Pentandione [5]
- Pharmaceuticals [7]
e.g. barbiturates, amphetamines, diuretics, antihistamines, narcotics etc. [7]
- Phenoxyacetic acid herbicides [8, 9]



Storage

The solutions should always be freshly made up.

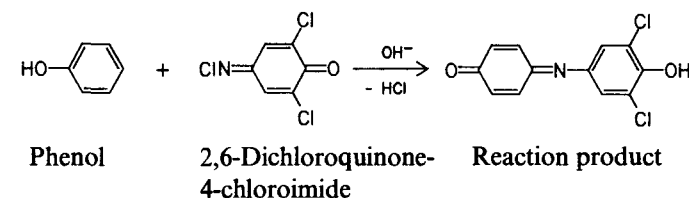
Caution: 2,6-dichloroquinone-4-chloroimide can decompose exothermically (5); it should, therefore, only be stored in small quantities in the refrigerator!

Substances

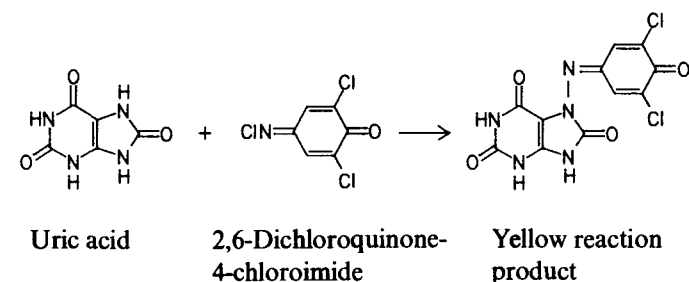
2,6-Dichloroquinone-4-chloroimide
Sodium hydrogen carbonate
Dimethyl sulfoxide (DMSO)
Chloroform
Ethanol

Reaction

2,6-Dichloroquinone-4-chloroimide, which can be replaced by 2,6-dibromoquinone-4-chloroimide (qv.) reacts preferentially with phenols and anilines which are not substituted in the *p*-position [2].



Uric acid couples with 2,6-dichloroquinone-4-chloroimide in position N-7 to yield a yellow dyestuff.



Preparation of Reagent

Dipping solution Dissolve 0.1 g 2,6-dichloroquinone-4-chloroimide in 10 ml dimethyl sulfoxide saturated with sodium hydrogen carbonate. Finally make up to 100 ml with chloroform.

Spray solution Dissolve 1 to 2 g 2,6-dichloroquinone-4-chloroimide in 100 ml ethanol [5, 6, 11] or toluene [8, 9] and filter, if necessary.

Method

The chromatogram is dried in a stream of cold air and heated to 110°C for 10 min, after cooling it is immersed in the reagent solution for 5 s or sprayed with it and then heated to 110°C for 2 min.

Note: If the spray solution or a nonbasic dipping solution is employed for detection then it is advisable to spray afterwards with a 10% aqueous solution of sodium carbonate or a 2% solution of borax in ethanol – water (1 + 1). It is often possible to achieve the required basicity by placing the chromatogram in a twin-trough chamber one of whose troughs contains 5 ml 25% ammonia. This is not suitable for the Chiralplate® (MACHEREY-NAGEL) because in this case the plate background acquires a dark violet coloration.

In general chromatogram zones of differing colors are formed even without warming. Thus, gallates produce brown zones, butylhydroxytoluene orange ones and butylhydroxyanisole blue ones on a light background [11]. The detection sensitivity is usually between 0.2 and 0.5 µg substance per chromatogram zone; *o*-phenylphenol and cephaeline can be detected 10 times more sensitively. Despite its free amino group serotonin does not produce a coloration [1].

The reagent can be employed on silica gel, kieselguhr, Si 50 000, NH₂, cellulose and polyamide layers.

Procedure Tested

Isoquinoline Alkaloids [12]

Method	Ascending, one-dimensional double development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Chloroform – methanol (15 + 5).
Migration distance	2 × 5 cm
Running time	2 × 12 min

Detection and result: The chromatogram was dried for 5 min in a stream of warm air, immersed in the dipping solution for 5 s and then heated to 110°C for 2 min.

Cephaeline (*hR_f* 40–45) produces a blue color spontaneously immediately on immersion, while emetine (*hR_f* 60–65) only does so on heating; on storage this color slowly changes to brown (background light brown). The detection limits for both substances are ca. 10 ng per chromatogram zone.

In situ quantitation: The absorption photometric analysis was made at $\lambda = 550$ nm (Fig. 1).

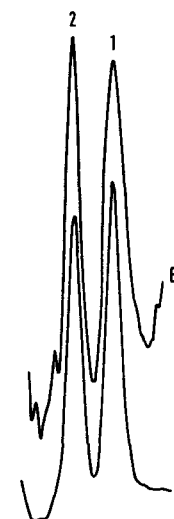


Fig. 1: Absorption scans of the pure substances (ca. 500 ng of each) cephaeline and emetine (A) and an extract of *Ipecacuanhae Radix* (B): cephaeline (1), emetine (2).

References

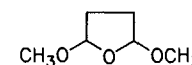
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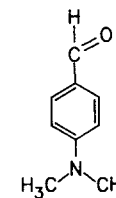
2,5-Dimethoxytetrahydrofuran – 4-Dimethylaminobenzaldehyde Reagent

Reagent for:

- Primary amines [1]
- Amino acids [1]
- Benzodiazepines [1]
- Panthenol [1]



$C_6H_{12}O_3$
 $M_r = 132.16$
 2,5-Dimethoxy-
 tetrahydrofuran



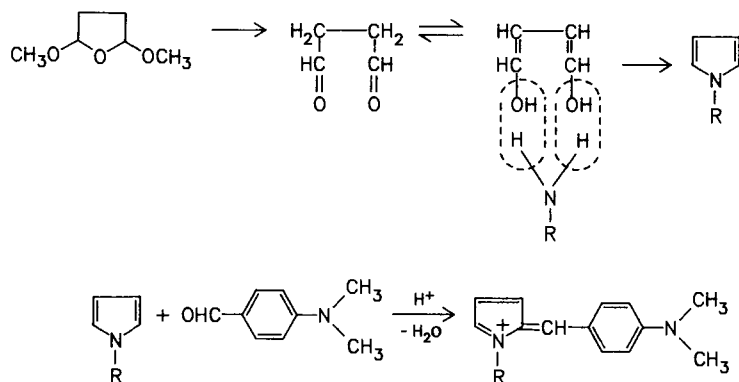
$C_9H_{11}NO$
 $M_r = 149.19$
 4-Dimethylamino-
 benzaldehyde

Preparation of the Reagent

- Dipping solution I** Mix 1 ml 2,5-dimethoxytetrahydrofuran with 99 ml glacial acetic acid [1] or ethyl acetate – glacial acetic acid (95 + 5).
- Dipping solution II** Dissolve 2 g 4-dimethylaminobenzaldehyde in 100 ml of a mixture of glacial acetic acid and 32% hydrochloric acid (85 + 15).
- Storage** Dipping solution I may be kept for several days and dipping solution II for several weeks at room temperature.
- Substances** 2,5-Dimethoxytetrahydrofuran
 4-Dimethylaminobenzaldehyde
 Acetic acid (glacial acetic acid)
 Hydrochloric acid (32%)
 Ethyl acetate

Reaction

Dimethoxytetrahydrofuran forms pyrrole derivatives with primary amines, these derivatives then condense with 4-dimethylaminobenzaldehyde in acid milieu to yield colored products [1]:



Method

The chromatogram is freed from mobile phase in a stream of warm air, dipped in dipping solution I for 3 s, then heated to 120°C for 5–10 min, cooled to room temperature and then immersed in dipping solution II for 3 s. The final drying of the chromatogram should take place in a stream of cold air in order to avoid strong background coloration.

Red-violet chromatogram zones on a weakly ochre-colored background are yielded within a few seconds (< 30 s) on silica gel layers.

Note: Traces of ammonia left by the mobile phase should be completely removed from the chromatograms before the reagent is applied in order to avoid strong background coloration. The dipping solutions may also be applied as spray solutions. Secondary amines, amides, pyrimidines and purines do not react with the reagent [1]. In the case of benzodiazepines only those substances react which

contain the structural element $\begin{array}{c} \text{O} \\ \parallel \\ \text{R}-\text{C} \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C}=\text{N} \\ | \end{array}$, such as, for example, diazepam or

nitrazepam; other benzodiazepines, such as, for example, chlorodiazepoxide or medazepam do not react [1]. The detection limits are at 5–50 ng for amino acids and benzodiazepines and at 500 ng substance per chromatogram zone for panthenol [1].

The reagent can be applied to silica gel, kieselguhr, Si 50 000, RP and CN layers. The detection sensitivity is reduced by a factor of 100 on cellulose layers; NH_2 layers are not suitable, as would be expected.

Occasionally, instead of dipping solution I it may be advisable to employ the following alternative dipping solution Ia mixed with citrate buffer in order to increase the detection sensitivity (e.g. with nitrazepam) [1].

Dipping solution Ia: Mix 1 ml 2,5-dimethoxytetrahydrofuran with 99 ml glacial acetic acid — citrate buffer, pH = 6.6 (1 + 2).

Citrate buffer solution: Dissolve 210 g citric acid in 400 ml caustic soda solution ($c = 5 \text{ mol/l}$) and make up to 1 l with water. Mix 530 ml of this solution with 470 ml caustic soda solution ($c = 1 \text{ mol/l}$) and adjust to pH 6.6 with caustic soda solution or citric acid [1].

Procedure Tested

Amino acids [2]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	2-Propanol — glacial acetic acid — water (16 + 3 + 3).
Migration distance	5 cm
Running time	55 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in reagent solution I for 3 s, heated to 120°C for 5–10 min, cooled to room temperature and finally immersed in reagent solution II for 3 s. It was then dried in a stream of cold air.

Glycine (hR_f 20–25), DL-alanine (hR_f 30–35), DL-valine (hR_f 45–50) and L-leucine (hR_f 55–60) yielded red-violet chromatogram zones on a pale yellow background. The detection limits for these amino acids were 5 ng substance per

chromatogram zone. With the exception of glycine the detection sensitivity was greater than with ninhydrin reagent.

In situ quantitation: Direct quantitative analysis was performed in reflectance at $\lambda = 580 \text{ nm}$ (Fig. 1).

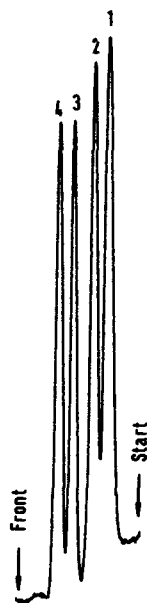


Fig. 1: Scanning curve of a chromatogram track with 100 ng per chromatogram zone of the amino acids glycine (1), alanine (2), valine (3), leucine (4).

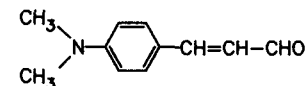
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4-Dimethylaminocinnamaldehyde — Hydrochloric Acid Reagent

Reagent for:

- Primary amines [1, 2, 9]
- Hydrazines [3]
- Indoles [4, 5, 13–15]
- Organoarsenic compounds [6]
- Urea [7, 8] and thiourea derivatives [9]
- Biotin [7]
- Sulfonamides [10]
- Pyrroles



$\text{C}_{11}\text{H}_{13}\text{NO}$ HCl
 $M_r = 175.23$ $M_r = 36.46$
 4-Dimethylamino-
 cinnamaldehyde

Preparation of the Reagent

Dipping solution Dissolve 0.5 g 4-dimethylaminocinnamaldehyde in 50 ml hydrochloric acid ($c = 5 \text{ mol/l}$) and make up to 100 ml with ethanol.

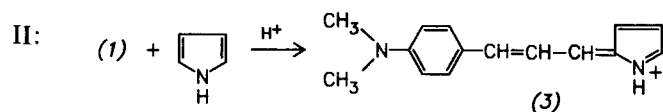
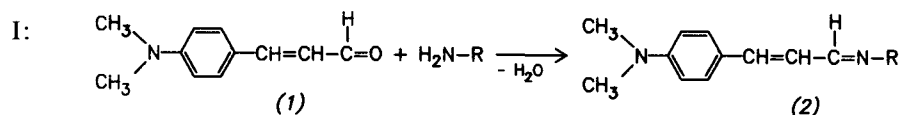
Spray solution Dilute the dipping solution 1 + 1 with ethanol for spraying.

Storage Both solutions may be stored for several days in the refrigerator.

Substances 4-Dimethylaminocinnamaldehyde
 Ethanol
 Hydrochloric acid 5 mol/l Combi-Titrisol

Reaction [11]

4-Dimethylaminocinnamaldehyde reacts with primary amines to form colored or fluorescent SCHIFF's bases (I). Pyrroles react with the reagent to form colored or fluorescent condensation products (II):



Method

The chromatogram is freed from mobile phase and dipped in the dipping solution for 1 s or uniformly sprayed with spray solution and then heated to 100°C for ca. 10 min. Colored zones are produced on a colorless to yellow background, after being left in the air for ca. 5 min they fluoresce greenish-yellow under long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: Sulfuric acid (4%) can also be employed in place of hydrochloric acid [3]. If ammoniacal mobile phases are employed the ammonia should be removed completely (e.g. heat to 105°C for 10 min) before dipping or spraying; otherwise background discoloration can occur. The addition of titanium(III) chloride to the reagent allows also the staining of aromatic nitro compounds [6].

Aminoglycoside antibiotics and β -substituted indoles are stained red. Pyrrole derivatives with free β -positions react at room temperature to yield blue-colored zones [11]. Exposure to the vapors of *aqua regia* deepens the colors. This reaction sometimes produces fluorescence [3]. The detection limit for monomethylhydrazine is 200 pg per chromatogram zone [3].

Silica gel, cellulose [3, 13] and ion exchanger layers are amongst the stationary phases that can be employed.

Procedure Tested

Gentamycin Complex [1, 12]

Method Ascending, one-dimensional development at 10–12°C in a twin-trough chamber with 5 ml conc. ammonia solution in the trough containing no mobile phase (chamber saturation 15 min).

Layer	HPTLC plates Silica gel 60 (MERCK), prewashed by triple development with chloroform — methanol (50 + 50) and drying for 30 min at 110°C.
Mobile phase	Chloroform — ethanol — ammonia solution (25%) (10 + 9 + 10); the <i>lower organic phase</i> was employed.
Migration distance	5 cm
Running time	20 min

Detection and result: The chromatogram was freed from mobile phase (NH_3 !) in a stream of cold air for 45 min. It was then immersed in the dipping solution for 1 s and heated to 100°C for ca. 20 min. The chromatograms could be further treated with ammonia vapor if a colored background was found to be troublesome. The pale red zones then became bright red spots and the background frequently

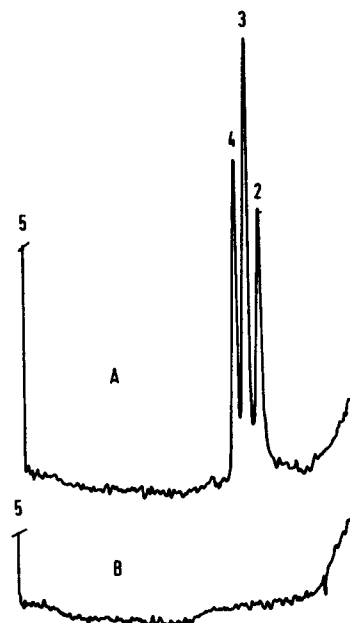


Fig. 1

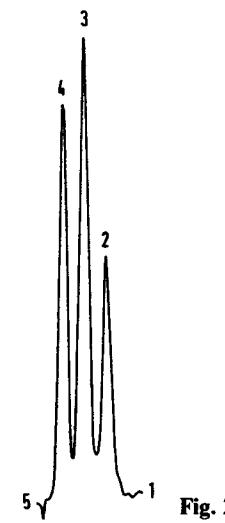


Fig. 2

Fig. 1: Absorption scan of a chromatogram track (A) of a gentamycin standard (600 ng gentamycin C complex) and of an accompanying blank (B). Start (1), gentamycin C_1 (2), gentamycin C_2 and C_{2a} (3), gentamycin C_{1a} (4), solvent front (5).

Fig. 2: Fluorescence scan of a gentamycin C complex. Peak order and amount applied as in Figure 1.

acquired a strong yellow coloration. Greenish-yellow fluorescent zones appeared after allowing to lie in the air for ca. 5 min.

In situ quantitation: Gentamycins could be both absorption photometrically and fluorimetrically quantitated.

The absorption scans were made at a wavelength of $\lambda = 505$ nm (Fig. 1). The limit of detection was 100 ng gentamycin C complex. The best conditions for fluorimetric determination (Fig. 2) were excitation at $\lambda_{\text{exc}} = 313$ nm and detection at $\lambda_{\text{fl}} > 390$ nm.

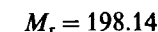
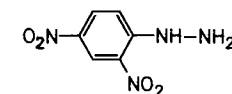
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2,4-Dinitrophenylhydrazine Reagent

Reagent for:

- Free aldehyde and keto groups [1–3]
e.g. in aldoses and ketoses
- *o*- and *m*-Dihydroxybenzenes,
dinitrophenols [1]
- Dehydroascorbic acid [4]
- Alkaloids [5, 6]
- Zearalenone [7, 8]
- Flavonoids
e.g. silymarin, silydianine [9]
- Phospholipids [10]
- Prostaglandins [11, 12]
- Valepotriates [13]

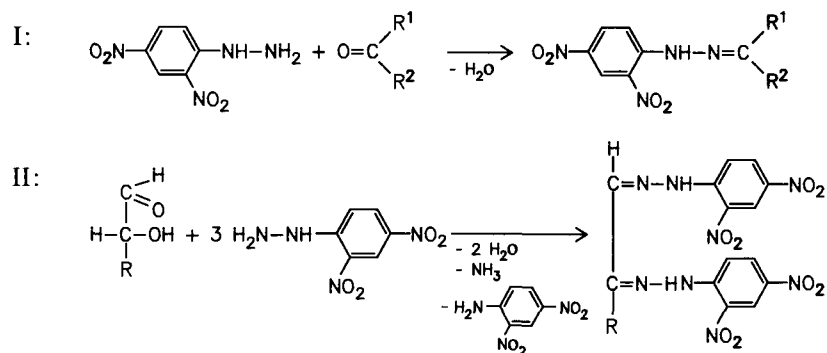


Preparation of the Reagent

- | | |
|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| Dipping solution | Dissolve 75 mg 2,4-dinitrophenylhydrazine in a mixture of 25 ml ethanol and 25 ml <i>ortho</i> -phosphoric acid (85%). |
| Spray solution | Dissolve 100 mg 2,4-dinitrophenylhydrazine in a mixture of 90 ml ethanol and 10 ml conc. hydrochloric acid. |
| Storage | Both solutions may be stored for several days in the refrigerator. |
| Substances | 2,4'-Dinitrophenylhydrazine
Hydrochloric acid (32%)
Hydrochloric acid (0.5 mol/l)
Ethanol
<i>ortho</i> -Phosphoric acid (85%) |

Reaction

2,4-Dinitrophenylhydrazine reacts with carbonyl groups with the elimination of water to yield hydrazones (I) and with aldoses or ketoses to yield colored osazones (II).



Method

The chromatogram is freed from the mobile phase in a current of warm air (2 to 10 min), immersed in the dipping solution for 2 s or sprayed evenly with the spray solution and then dried in a stream of warm air (or 10–20 min at 110°C).

Substances containing aldehyde or keto groups yield yellow to orange-yellow chromatogram zones on an almost colorless background [1, 11]. Silymarin appears red-blue and silydianine ochre-colored [9].

Note: The spray reagent can be made up with sulfuric acid instead of hydrochloric acid [9]. In contrast to other prostaglandins containing carbonyl groups which yield yellow-orange colored chromatogram zones, some of them without heating, 6-oxo-PGF₁ does not react even when heated [11].

The reagent can be employed on silica gel and cellulose layers.

Procedure Tested

Dehydroascorbic Acid [4]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Acetone – toluene – formic acid (60 + 30 + 10).
Migration distance	6 cm
Running time	11 min

Detection and result: The chromatogram was freed from mobile phase in a current of warm air (2 min), immersed in the reagent solution for 5 s and dried in a current of warm air or at 110°C for 10 min.

Dehydroascorbic acid (*hR_f* 45–50) appears as a yellow zone on a colorless background.

The detection limit for dehydroascorbic acid is 10 ng per chromatogram zone.

In situ quantitation: The quantitative analysis is performed by measuring the absorption of the chromatogram zone in reflectance at $\lambda = 440 \text{ nm}$ (Fig. 1).

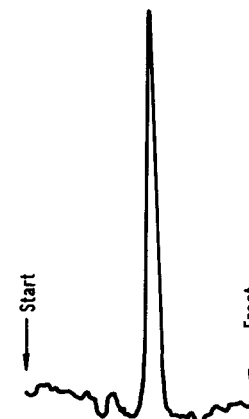


Fig. 1: Absorption scan of a chromatogram containing 200 ng dehydroascorbic acid per chromatogram zone.

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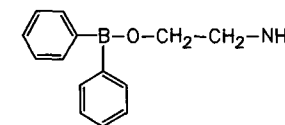
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Diphenylboric acid-2-aminoethyl Ester Reagent

(Flavone Reagent According to NEU)

Reagent for:

- Flavonoids [1–16, 21]
- Penicillic acid [17, 18]
- Carbohydrates [19]
e.g. glucose, fructose, lactose
- Anthocyanidines [20]
- Hydroxy- and methoxycinnamic acid [20]



$C_{14}H_{16}BNO$
 $M_r = 225.10$

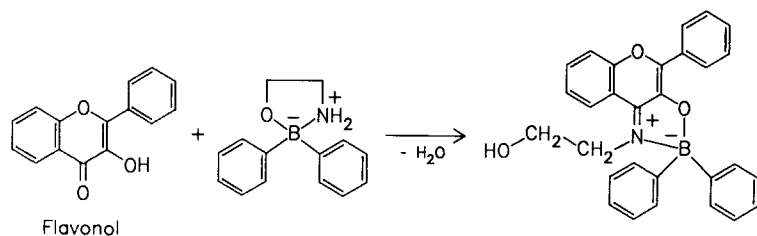
Preparation of the Reagent

- Dipping solution** Dissolve 1 g diphenylboric acid-2-aminoethyl ester (diphenylboric acid β -aminoethyl ester, diphenylboryloxyethylamine, “Naturstoffreagenz A”, Flavognost®) in 100 ml methanol.
- Storage** The reagent solution may be kept for several days in the refrigerator. However, it should always be freshly prepared for quantitative analyses.
- Substances** Naturstoffreagenz A
Methanol

Reaction

Diphenylboric acid-2-aminoethyl ester reacts to form complexes with 3-hydroxyflavones* with bathochromic shift of their absorption maximum.

* Hohaus, E.: Private communication, Universität-GH Siegen, Analytische Chemie, D-5900 Siegen, 1987.



Method

The chromatograms are heated to 80°C for 10 min and allowed to cool in a desiccator for 10 min, they are then immersed in the dipping solution for 1 s or sprayed with it, dried in a stream of warm air for ca. 1 min and stored in the desiccator for 15 min. Finally, they are immersed for 1 s in a solution of liquid paraffin – *n*-hexane (1 + 2) to stabilize the fluorescence and – after drying for 1 min in a stream of warm air – irradiated for ca. 2 min with intense long-wavelength UV light ($\lambda = 365$ nm).

Substances are produced with a characteristic fluorescence in long-wavelength UV light ($\lambda = 365$ nm).

Note: A 5% solution of polyethylene glycol 4000 in ethanol can be sprayed onto the chromatogram [2, 4] for the purpose of increasing and stabilizing the fluorescence instead of dipping it in liquid paraffin – *n*-hexane (1 + 2). If this alternative is chosen the plate should not be analyzed for a further 30 min since it is only then that the full intensity of the fluorescence develops [6].

In order to detect penicillic acid (detection limit: ca. 5 ng) the plate is heated to 110°C for 15 min after it has been sprayed with reagent; this causes penicillic acid to produce pale blue fluorescent zones [17, 18].

In long-wavelength UV light ($\lambda = 365$ nm) carbohydrates, e.g. glucose, fructose and lactose, yield pale blue fluorescent derivatives on a weakly fluorescent background. In situ quantitation can be performed at $\lambda_{\text{exc}} = 365$ nm and $\lambda_{\text{fl}} = 546$ nm (monochromatic filter M 546) [19]. Further differentiation can be achieved by spraying afterwards with *p*-anisidine-phosphoric acid reagent [8].

The reagent can be employed on silica gel, kieselguhr, cellulose, RP, NH₂ and polyamide-11 layers.

Procedure Tested

Flavonoids [21]

Method

Ascending, one-dimensional development in a trough chamber with chamber saturation. After application of the samples but before development the layer was conditioned for 30 min over water.

Layer

HPTLC plates Silica gel 60 (MERCK). The layer was pre-washed by developing once in methanol and then drying at 110°C for 30 min.

Mobile phase

Ethyl acetate – formic acid – water (85 + 10 + 15).

Migration distance

6 cm

Running time

30 min

Detection and result: The developed chromatogram was heated to 80°C for 15 min; the warm plate was sprayed first with “Flavone Reagent” (3% in methanol)

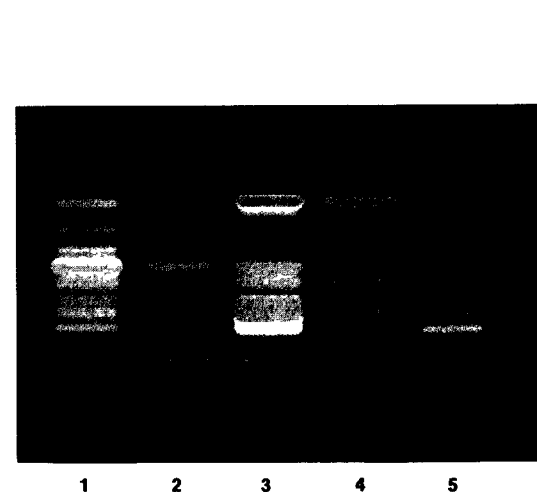


Fig. 1

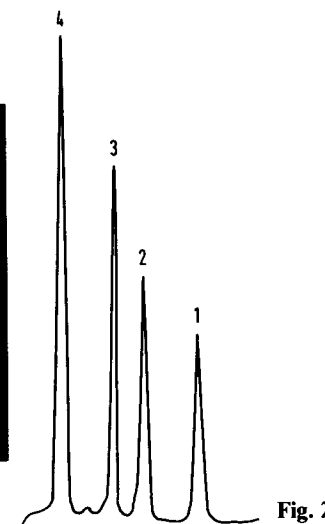


Fig. 2

Fig. 1: Chromatography of flavonoids. 1. Extract. Solidaginis; 2. Rutin – chlorogenic acid – isoquercitrin – quercitrin; 3. Extract. Hyperici; 4. Hyperosid – quercetin-3-arabinosid – hypericin – quercetin; 5. Extract. Betulae

Fig. 2: Fluorescence scan of a chromatogram track with 30 ng substance per chromatogram zone of rutin (1), hyperoside (2), quercitrin (3) and quercetin (4).

and then immediately with polyethylene glycol 6000 (5% in ethanol). After drying at room temperature for 30 min the chromatogram was inspected under long-wavelength UV light ($\lambda = 365$ nm).

Colored substance zones were obtained which could be analyzed quantitatively. The (visual) detection limits were: hypericin 1 ng, rutin and chlorogenic acid 5 ng, hyperoside – quercetin 10 ng per mm chromatogram zone. The detection limits for densitometric analysis are between 20 and 50% of those for visual detection.

The following hR_f values were obtained: quercetin 90–95, hypericin 75–80, quercitrin 60–65, quercetin-3-arabinoside 55–60, isoquercitrin 45–50, hyperoside 40–45, chlorogenic acid 30–35, rutin 20–25 (Fig. 1).

In situ quantitation: The in situ fluorescence determination was carried out by excitation at $\lambda_{exc} = 436$ nm and detection at $\lambda_{fl} = 546$ nm (monochromate filter M 546) (Fig. 2).

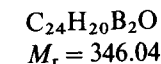
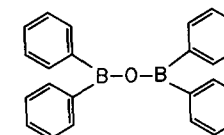
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Diphenylboric Anhydride Reagent (DBA Reagent)

Reagent for:

- 2-(2-Hydroxyphenyl)benztriazole derivatives (UV absorber in plastics)*



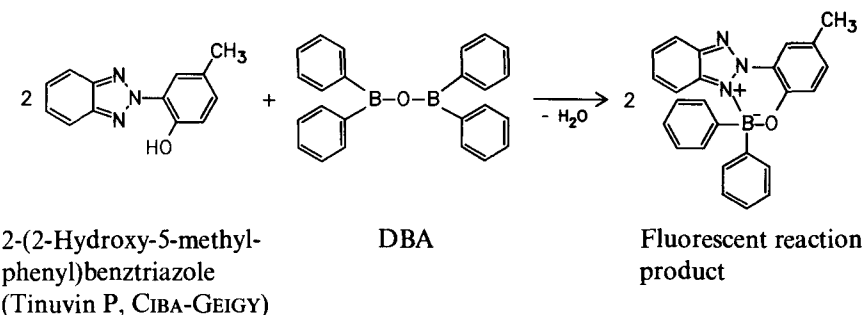
Preparation of the Reagent

Dipping solution	Dissolve 350 mg diphenylboric anhydride (DBA) in 100 ml methanol.
Storage	DBA should be stored dry, cool and protected from light. The solution should be freshly made up every day.
Substances	Diphenylboric anhydride Methanol

Reaction

The reaction takes place with the formation of fluorescent chelates according to the following scheme:

* Hohaus E.: Private communication, Universität-GH Siegen, Analytische Chemie, D-5900 Siegen, 1985.



Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed for 1 s in the reagent solution or sprayed evenly with it and dried in a stream of cold air.

Yellow-green fluorescent zones are formed on a dark background in long-wavelength UV light ($\lambda = 365$ nm).

Note: Since neither the DBA reagent nor 2-(2-hydroxyphenyl)benzotriazole are intrinsically fluorescent the chromatogram is not affected by interfering signals.

The reagent can be employed, for example, on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

2-(2-Hydroxy-5-methylphenyl)benzotriazole*

Method	Ascending, one-dimensional development in a twin-trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 (MERCK).

* Hohaus, E., Monien, H., Overhoff-Pelkner, A.-P.: private communication, Universität-GH Siegen, Analytische Chemie, D-5900 Siegen 1985.

Mobile phase Carbon tetrachloride.

Migration distance 10 cm

Running time ca. 12 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in the reagent solution for 1 s and dried in a stream of warm air.

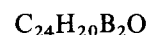
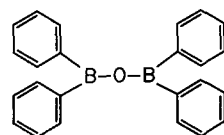
Under long-wavelength UV light ($\lambda = 365$ nm) the 2-(2-hydroxyphenyl)-benzotriazoles yielded yellow-green fluorescent chromatogram zones, which were, in the cases of Tinuvin P (hR_f 20–25) and Tinuvin 343 (2-[2-hydroxy-3-(1-methylpropyl)-5-*tert*-butylphenyl]benzotriazole; hR_f 45–50), suitable for quantitation.

In situ quantitation: Quantitative determination is performed fluorimetrically ($\lambda_{\text{exc}} = 365$ nm, $\lambda_{\text{fl}} = 535$ nm), the detection limits are 250 ng substance per chromatogram zone.

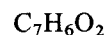
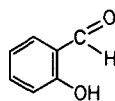
Diphenylboric Anhydride – Salicylaldehyde Reagent (DOOB Reagent)

Reagent for:

- Primary (!) amines
e.g. alkyl amines [1–3]
lipid amines [4]
 α , ω -diamines [5, 6]
polyamines [6]
alkanol amines [7]
subst. anilines [8]
aminoglycoside
antibiotics [9, 10]
biogenic amines [11]
hydrazines



$M_r = 346.04$
Diphenylboric
anhydride



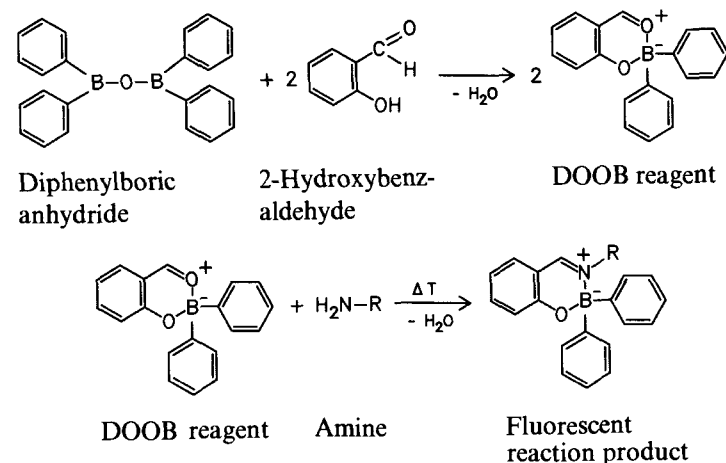
$M_r = 122.12$
Salicylaldehyde

Substances

Diphenylboric anhydride
2-Hydroxybenzaldehyde
Chloroform

Reaction

The DOOB reagent, which is formed by reaction of diphenylboric anhydride with salicylaldehyde, yields fluorescent reaction products with primary amines [1].



Method

The chromatogram is freed from mobile phase (stream of warm air, 15 min), immersed for 2 s in the reagent solution after cooling to room temperature and heated to 110–120°C for 10–20 min. The chromatogram is then briefly immersed in liquid paraffin – *n*-hexane (1 + 6) in order to enhance and stabilize the fluorescence.

After evaporation of the hexane blue fluorescent chromatogram zones are visible on a dark background under long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: The dipping solution may also be used as spray solution [1]. The reagent may be applied to RP layers; it is not suitable for amino phases.

Preparation of the Reagent

Dipping solution Dissolve 35 mg diphenylboric anhydride (DBA) and 25 mg salicylaldehyde (= 2-hydroxybenzaldehyde) in 100 ml chloroform. This results in the formation of 2,2-diphenyl-1-oxa-3-oxonia-2-boratanaphthalene (DOOB).

Storage DBA solutions should only be stored for a short time even in the refrigerator. On the other hand, DOOB reagent solution in bottles with ground-glass stoppers may be stored in the refrigerator for at least 2 weeks.

Procedure Tested

Netilmicin and Gentamycin-C Complex [10]

Method	Ascending, one-dimensional development in a HPTLC-trough chamber with chamber saturation.
Layer	KC8F or KC18F plates (WHATMAN).
Mobile phase	0.1 mol/l LiCl in 32% ammonia solution — methanol (25 + 5).
Migration distance	10 cm
Running time	30 min

Detection and result: The chromatogram was dried in a stream of warm air for 15 min, cooled and immersed in the reagent solution for 2 s. It was then heated to 110–120°C for 10–20 min, allowed to cool to room temperature and immersed for 2 s in liquid paraffin — *n*-hexane (1 + 6) to enhance and stabilize the fluores-

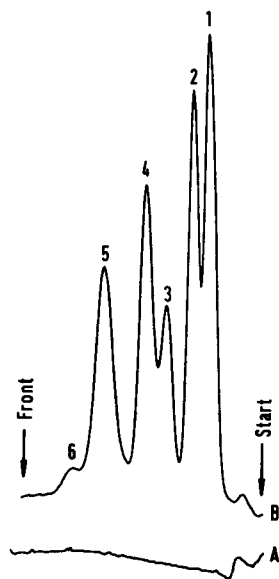


Fig. 1: Fluorescence scan of a blank track (A) and of a chromatogram track (B) with 500 ng netilmicin and 1 µg gentamycin C complex as sample. Netilmicin (1), gentamycin C₁ (2), gentamycin C_{2a} (3), gentamycin C₂ (4), gentamycin C_{1a} (5), impurity in netilmicin (6).

cence. After the evaporation of the *n*-hexane the netilmicin and the gentamycin components appeared as blue fluorescent chromatogram zones on a dark background under long-wavelength UV light ($\lambda = 365$ nm). The same applied to neomycin. The detection limits were 10 ng per chromatogram zone for netilmicin and 15–20 ng per chromatogram zone for the various gentamycin components.

Note: If netilmicin is to be chromatographed alone it is recommended that the methanol content of the mobile phase be increased (e.g. to 23 + 7), in order to increase the value of the R_F . The detection limit for the substances in the application tested was more sensitive using DOOB reagent on RP layers than when NBD chloride, fluorescamine or *o*-phthalaldehyde were employed. The derivatives so formed were stable and still fluoresced after several weeks if they were stored in the dark.

In situ quantitation: Quantitative analysis was performed fluorimetrically ($\lambda_{exc} = 365$ nm, $\lambda_{fl} > 430$ nm) (Fig. 1).

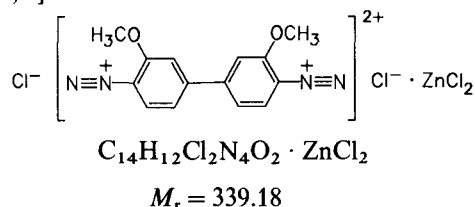
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Fast Blue Salt B Reagent

Reagent for:

- Phenols
e.g. *n*-alkylresorcinol homologues [1]
thymol derivatives [2]
hydroxyanthraquinones [3]
ratanhia phenols [4]
cardol, anacardol [5]
phloroglucinol derivatives [6, 7]
- Tannins [8, 9]
- α - and γ -Pyrone derivatives
e.g. coumarins [10]
flavonols [11]
- Phenolcarboxylic acids [12]
- Cannabinoids [13–18]
- Amines capable of coupling
e.g. carbamate pesticides [19]
pharmaceutical metabolites [20–22]



Preparation of Reagent

Dipping solution Dissolve 140 mg fast blue salt B in 10 ml water and make up to 40 ml with methanol. Add 20 ml of this solution with stirring to a mixture of 55 ml methanol and 25 ml dichloromethane.

Spray solution I Dissolve 0.1 to 5 g fast blue salt B in water [7, 8], 70% ethanol [17] or acetone-water (9 + 1) [14].

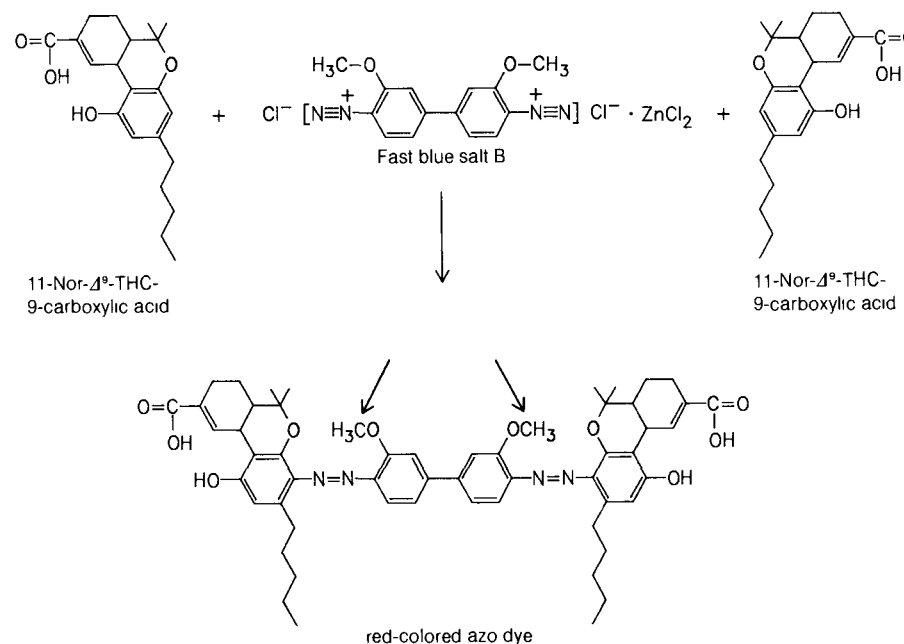
Spray solution II Caustic soda solution ($c = 0.1 \text{ mol/l}$)

Storage The reagent solutions should always be freshly made up and fast blue salt B should always be stored in the refrigerator.

Substances Fast blue salt B
Methanol
Dichloromethane
Sodium hydroxide solution (0.1 mol/l)

Reaction

Fast blue salt B couples best with phenols in alkaline medium, e.g. with 11-nor- Δ^9 -THC-9-carboxylic acid to yield the following red-colored product:



Method

The chromatograms are dried in a stream of warm air, then placed for 10–15 min in the empty half of a twin-trough chamber whose other trough contains 25 ml conc. ammonia solution (equilibrate for 60 min!) and then immediately immersed in the dipping reagent for 5 s [18] and dried for 5 min in a stream of warm air.

Alternatively the dried chromatograms can be homogeneously sprayed with spray solutions I and II consecutively.

Chromatogram zones of various colors are produced (yellow, red, brown and violet) on an almost colorless background.

Note: The detection is not affected if the dipping solution exhibits a slight opalescent turbidity. Fast blue salt BB [18] or fast blue salt RR [18, 19] can be employed in the reagent in place of fast blue salt B. It is occasionally preferable not to apply spray solutions I and II separately but to work directly with a 0.1% solution of fast blue salt B in caustic soda solution ($c = 1–2$ mol/l) [13, 15] or in 0.5% methanolic caustic potash [3].

A dipping solution consisting of 0.2% fast blue salt B in hydrochloric acid ($c = 0.5$ mol/l, immersion time: 30 s) has been reported for the detection of resorcinol homologues [1].

Detection limits of 250 ng per chromatogram zone have been reported for THC-11-carboxylic acid [15] and of 500 ng per chromatogram zone for carbamate pesticides [19].

The reagent can be employed on cellulose, silica gel and polyamide layers [11]; kieselguhr, RP and Si 50 000 layers are also suitable.

Caution: Fast blue salt B may be carcinogenic [23].

Procedure Tested

THC Metabolites in Urine after Consumption of Hashish [18]

Method

Ascending, one-dimensional multiple development method (stepwise technique, drying between each run) in two mobile phase systems in a twin-trough chamber without chamber saturation (equilibration: 30 min at 20–22 °C) at a relative humidity of 60–70%.

Layer	HPTLC plates Silica gel 60 F _{254s} (MERCK) or HPTLC plates Silica gel 60 WRF _{254s} (MERCK).
Mobile phase 1	Toluene – ethyl acetate – formic acid [16 + 4 + 0.5 (0.4*)].
Mobile phase 2	Toluene – ethyl acetate – formic acid [17 + 3 + 0.25 (0.2*)].
Migration distances	Eluent 1: 7.5 mm, 15.0 mm and 22.5 mm Eluent 2: 30.0 mm, 37.5 mm and 45 mm

Detection and result: The chromatogram was dried in a stream of warm air for 5 min (it is essential to remove all traces of formic acid) and then placed for 10–15 min in the empty half of a twin-trough chamber whose other trough contained 25 ml conc. ammonia solution (equilibrated for 60 min!). It was immersed immediately afterwards in the dipping reagent for 5 s and dried in a stream of warm air for 5 min.

In visible light the chromatogram zones were red on an almost colorless background (Fig. 1), they slowly turned brownish on exposure to air and appeared

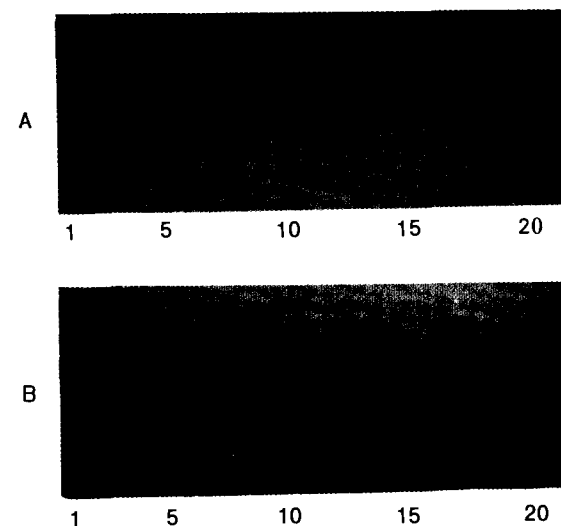


Fig. 1: Chromatograms of urine samples containing THC metabolites; detection with fast blue salt RR (A) and fast blue salt B (B). Track A₁ and B₁: metabolite-free urines; tracks A₁₆ and B₁₅ represent ca. 60 ng total cannabinoids per ml urine (determined by RIA).

* These quantities apply to the WRF_{254s} layer.

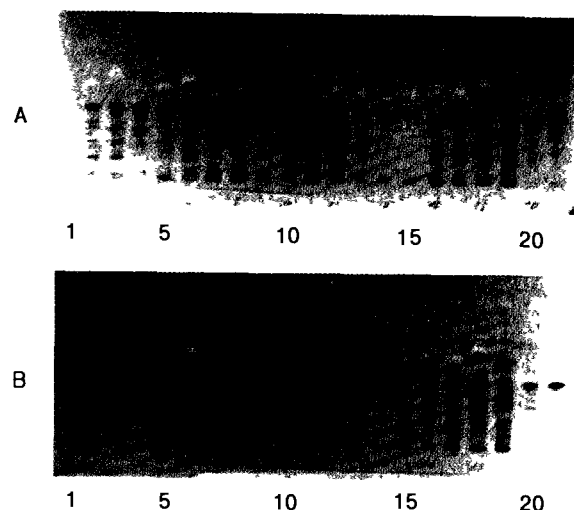


Fig. 2: Chromatogram from Fig. 1 observed under long-wavelength UV light ($\lambda = 365$ nm).

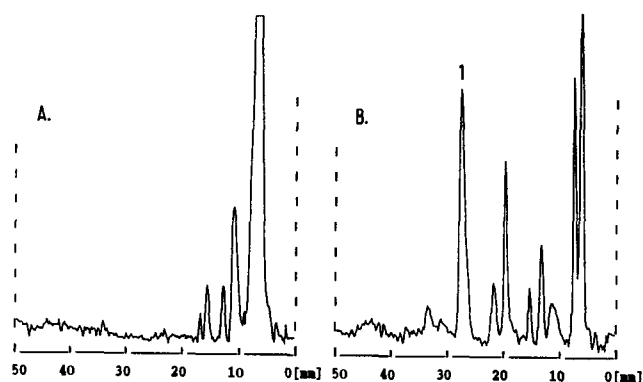


Fig. 3 Reflectance scan of a urine free from THC metabolites (A) and one containing THC metabolites (B): Peak 1 corresponds to ca. 100 ng 11-nor- Δ^9 -THC-9-carboxylic acid per ml urine

under UV light on the indicator-containing layer as dark blue zones on a pale blue fluorescent background.

Additional dipping in a 0.001% solution of dansyl semipiperazide or bis-dansyl piperazide in dichloromethane — liquid paraffin (75 + 25) stabilized the color of the chromatogram for a period of months.

Note: The alternative fast blue salt BB produced the most intensely colored chromatogram zones for visual analysis in daylight, while fluorescence quenching in UV light ($\lambda = 254$ nm) was greater with fast blue salt B and fast blue salt RR (Figs. 1 and 2).

In situ quantitation: The absorption photometric analysis was performed at $\lambda = 546$ nm (Fig. 3).

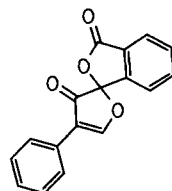
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Fluorescamine Reagent

Reagent for:

- Primary amines, amino acids [1 – 5]
e.g. histamine [4], sympathomimetics [6], catecholamines, indolamines [5], arylamines [7], gentamicins [8]
- Peptides [2, 5]
- Secondary amines [9]
- Sulfonamides [10 – 14]



$C_{17}H_{10}O_4$
 $M_r = 278.27$

Preparation of the Reagent

Dipping solution I Dissolve 10–50 mg fluorescamine (Fluram®) in 100 ml acetone [2, 9, 10].

Dipping solution II Make 10 ml triethylamine up to 100 ml with dichloromethane [2].

Spray solution I Dissolve 1.9 g *di*-sodium tetraborate decahydrate in 100 ml water and adjust the pH to 10.5 with caustic soda solution.

Spray solution II Dissolve 3.6 g *di*-sodium hydrogen phosphate dihydrate in 100 ml water and adjust the pH to 7.5 with phosphoric acid. Dissolve 2.5 g taurine in this.

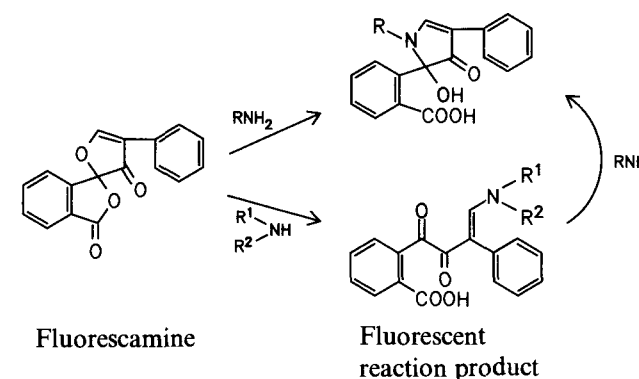
Storage The dipping solutions and the spray solutions may be stored for at least one week in the refrigerator [2].

Substances Fluorescamine
Triethylamine
Acetone
Dichloromethane

di-Sodium tetraborate decahydrate
Taurine
di-Sodium hydrogen phosphate dihydrate

Reaction

Fluorescamine reacts directly with primary amines to form fluorescent products. Secondary amines yield nonfluorescent derivatives which can be transformed into fluorescent products by a further reaction with primary amines.



Method

Primary amines: The chromatograms are dried for 10 min at 110°C, cooled to room temperature and then immersed in dipping solution I or sprayed evenly with it and then dried for a few seconds in the air.

Then they are immersed in dipping solution II for 1 s or sprayed with it.

Fluorescent blue-green chromatogram zones appear on a dark background in long-wavelength UV light ($\lambda = 365$ nm).

Secondary amines: The chromatograms are first freed from mobile phase and then sprayed evenly with spray solution I and heated to 110°C for 15 min. Then they are immersed in dipping solution I or sprayed with it, stored in the dark for 15 min, then sprayed with spray solution II and heated to 60°C for 5 min.

Sulfonamides: The chromatograms are freed from mobile phase and immersed in dipping solution I for 1 s or sprayed evenly with it [10, 11].

After ca. 15 min green-yellow fluorescent chromatogram zones appear on a dark background in long-wavelength UV light ($\lambda = 365$ nm).

Note: The pre- and post-treatment of the chromatograms with the basic triethylamine solution, which can be replaced by an alcoholic solution of sodium hydroxide [1, 4] or a phosphate buffer solution pH = 8.0 ($c = 0.2$ mol/l) [5], serves to stabilize the fluorescence of the amino derivatives [2]. A final spraying with methanolic hydrochloric acid ($c_{\text{HCl}} = 5$ mol/l) or 70% perchloric acid renders the detection reaction highly specific for histamine [4] and for catecholamines and indolamines [5].

The reagent can also be employed for prechromatographic derivatization by over-spotting [6] or dipping [5].

The detection limit for amines and sulfonamides lies in the low nanogram range.

The layers with which the reagent can be employed include silica gel, kieselguhr, Si 50000 and cellulose.

Procedure Tested

Primary Amines [15]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Cellulose (MERCK).
Mobile phase	1-Butanol — glacial acetic acid — water (15 + 5 + 5).
Migration distance	5 cm
Running time	25 min

Detection and result: The chromatogram was freed from mobile phase, immersed in dipping solution I for 1 s, dried briefly in a stream of cold air and then immersed for 1 s in dipping solution II. The layer was then dipped in a mixture of liquid paraffin — *n*-hexane (1 + 5) in order to enhance the sensitivity of the fluorescence by 25–40% and to stabilize it.

Glycine (hR_f 20–25), alanine (hR_f 35–40), valine (hR_f 55–60) and leucine (hR_f 65–70) appeared as blue-green fluorescent chromatogram zones in long-wave-

length UV light ($\lambda = 365$ nm); these zones could be quantitatively determined after 15 min (Fig. 1).

In situ quantitation: The fluorimetric determination was performed at $\lambda_{\text{exc}} = 365$ nm and $\lambda_{\text{fl}} > 460$ nm. The detection limits were 5–20 ng substance per chromatogram zone.

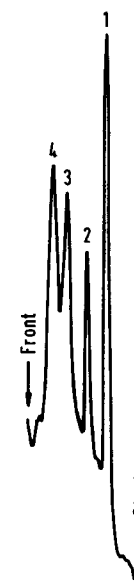


Fig. 1: Fluorescence scan of a chromatogram track with 200 ng of each substance per chromatogram zone. Glycine (1), alanine (2), valine (3), leucine (4).

References

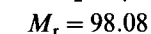
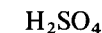
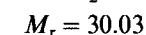
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Formaldehyde – Sulfuric Acid Reagent (Marquis' Reagent)

Reagent for:

- Aromatic hydrocarbons and heterocyclics [1]
- β -Blockers
e.g. pindolol, alprenolol, propranolol, oxprenolol, nadolol etc. [2, 3]
- Alkaloids
e.g. morphine, codeine, heroin
6-monoacetylmorphine [4]
- Amphetamines
e.g. 2,5-dimethoxy-4-bromoamphetamine [5]
- Methyl esters of fatty acids [6]
- Phenothiazines [7]
- Tannins [8]
- Guaifenesin [9]



Preparation of Reagent

Dipping solution Add 10 ml sulfuric acid (95 – 97%) carefully to 90 ml methanol. To this add 2 ml formaldehyde solution (37%).

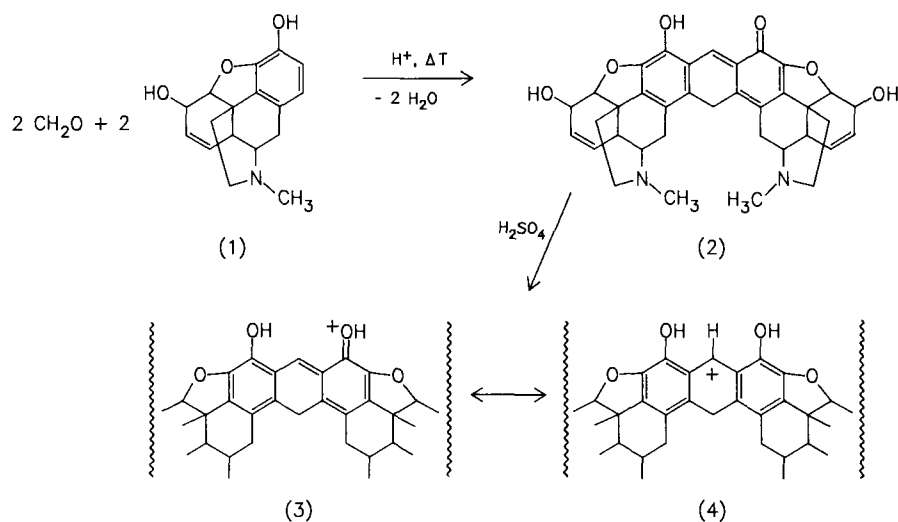
Spray solution Add 0.2 – 1 ml formaldehyde solution (37%) carefully to 10 ml conc. sulfuric acid [1 – 3].

Storage The dipping solution may be stored for ca. 4 weeks in the refrigerator.

Substances Formaldehyde solution ($\geq 37\%$)
Sulfuric acid (95–97%)
Methanol

Reaction

Morphine (1) reacts with formaldehyde in acidic solution to yield a cyclic ketoalcohol (2) which is transformed into the colored oxonium (3) or carbenium ion (4) in acidic conditions [10].



Method

The chromatograms are freed from mobile phase (5 min in a stream of warm air), immersed in the dipping solution for 4 s or sprayed evenly with it and then heated to 110°C for 20 min (the methyl esters of fatty acids are heated to 140°C for 10 min [6]).

Various colored chromatogram zones are formed on a pale pink background, some of them before heating. These zones frequently fluoresce in long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: The dipping reagent is to be preferred because of the strongly irritating effects of formaldehyde on the respiratory tract. Detection limits of ca. 10–40 ng have been reported for alkaloids [4] and 50 ng–1 μg for β -blockers [2, 3].

The reagent can be employed on silica gel, kieselguhr, Si 50 000 and aluminium oxide layers.

Procedure Tested

Morphine Alkaloids [4]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK) which had been pre-washed by developing once with chloroform – methanol (50 + 50) and then dried at 110°C for 20 min.
Mobile phase	Methanol – chloroform – water (12 + 8 + 2).
Migration distance	6 cm
Running time	20 min

Detection and result: The chromatogram was dried in a stream of warm air for 5 min, immersed in the dipping solution for 6 s and heated to 110°C for 20 min. After drying in a stream of cold air morphine (hR_f 25–30), 6-monoacetylmorphine (hR_f 40–45) and heroin (hR_f 50–55) yielded reddish chromatogram zones and codeine (hR_f 30–35) yielded blue chromatogram zones on a pale pink background.

If a quantitative fluorimetric analysis was to follow the chromatogram was then exposed to ammonia vapor for 20 min (twin-trough chamber with 25% ammonia in the vacant trough) and then immediately immersed for 2 s in a 20% solution of dioctyl sulfosuccinate in chloroform. After drying in a stream of cold air the morphine alkaloids investigated appeared under long-wavelength UV light ($\lambda = 365 \text{ nm}$) as pink to red fluorescent zones on a blue fluorescent background.

In situ quantitation: The absorption-photometric determination in a reflectance mode was performed at $\lambda = 330$ nm (detection limit ca. 40 ng per chromatogram zone). The fluorimetric analysis was carried out at $\lambda_{exc} = 313$ nm and $\lambda_{fl} > 560$ nm (detection limits: ca. 10 ng per chromatogram zone) (Fig. 1).

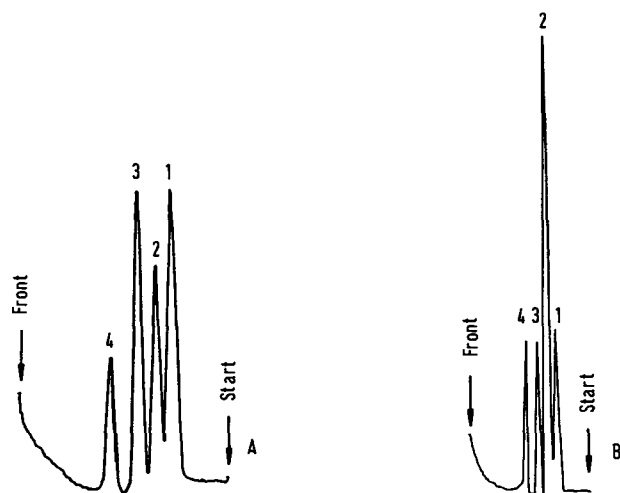


Fig. 1: Reflectance scan (A) and fluorescence scan (B) of a mixture of alkaloids with 725 ng (A) and 100 ng (B) substance per chromatogram zone. Morphine (1), codeine (2), 6-mono-acetylmorphine (3), heroin (4).

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Hydrochloric Acid Vapor Reagent

Reagent for:

- Antiepileptics
e.g. carbamazepine [1, 2]
primidone, phenytoin, phenylethylmalonamide, phenobarbital [3]
- Chalcones [4]
- pH indicators
e.g. 4-aminoazobenzene derivatives [5]
- Digitalis glycosides [6–10]
e.g. acetyldigoxin [6], digoxin [7, 9]
digitoxin [8]
- Carbohydrates, diazepam, testosterone [11]
- Alkaloids
e.g. papaverrubines [12]
- Anabolics
e.g. trenbolone [13–15]
- Chloroplast pigments [16]
- Bitter principles [17]
- Penicillic acid [18]

HCl
 $M_r = 36.46$

Preparation of Reagent

Reagent solution Place 10 ml conc. sulfuric acid in a twin-trough chamber and add 2 ml conc. hydrochloric acid dropwise and with care.

Storage The reagent should be made up fresh daily.

Substances Hydrochloric acid (32%)
Sulfuric acid (95–97%)

Reaction

The reaction mechanism has not yet been elucidated.

Method

Free the chromatogram from mobile phase (first in a stream of cold air for a few minutes, then at 100°C for 5 min), place in the free trough of the prepared twin-trough chamber for 5 min and then evaluate immediately in the case of chalcones or 4-aminoazobenzene derivatives. Digitalis glycosides and carbohydrates as well as diazepam and testosterone are first viewed after reheating (to 160–165°C for 15 min) [6, 7, 11] or after irradiation with unfiltered UV light from a mercury lamp [8]. In the case of antiepileptics the chromatogram is irradiated for 15 min with unfiltered UV light from a mercury lamp immediately after exposure to the HCl vapors and then inspected [1, 2].

Chalcones yield orange-red to brown-colored zones [4] as do 4-aminoazobenzene derivatives, but their colors begin to change after 10 min and slowly fade [5]. Penicillic acid is visible as a greyish-black zone [18].

Antiepileptics [3], carbohydrates, diazepam and testosterone [11] as well as digitalis glycosides are visible as light blue fluorescent zones on a dark background in long-wavelength UV light ($\lambda = 365$ nm); the blue fluorescence of the digitalis glycosides turns yellow after irradiation with UV light [7, 8]. Trenbolone fluoresces green; its detection limit in meat is 5 ppb [14].

Note: The reagent can be employed on silica gel, kieselguhr, Si 50 000 and RP layers [6]. The fluorescence intensities of the chromatogram zones can be increased by dipping in a solution of liquid paraffin in hexane or chloroform [8, 11].

Procedure Tested

Digitalis Glycosides [9]

Method Ascending, one-dimensional double development (stepwise technique) in a trough chamber with chamber saturation.

Layer HPTLC plates Silica gel 60 with concentrating zone (MERCK).

Application technique The samples were applied as bands (ca. 5 mm long) at right angles to the lower edge of the plate (that is parallel to the future direction of development), e.g. with the Linomat III (CAMAG).

Mobile phase 1. Chloroform;
2. Acetone – chloroform (65 + 35).

Migration distance Mobile phase 1: 7 cm
Mobile phase 2: 4 cm

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and exposed to hydrochloric acid gas (LINDE) in a Teflon autoclave (diameter 20 cm, height 8 cm). For this purpose the HPTLC plate was laid on a

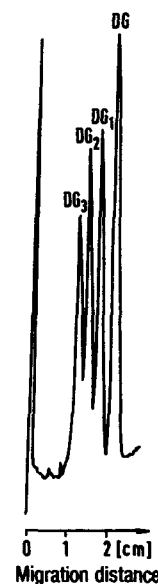


Fig. 1



Fig. 2

Fig. 1: Fluorescence scan of a mixture of digitalis glycosides. Digoxin (DG₃), digoxigenin bis-digitoxoside (DG₂), digoxigenin mono-digitoxoside (DG₁), digoxigenin (DG).

Fig. 2: Fluorimetric analysis of various quantities of digoxin for evaluation of the detection limit.

Teflon support with its glass side facing the gas inlet; the autoclave was evacuated (10 min, < 1 mbar), carefully filled with HCl gas (1 bar) and heated to 110°C for 15 min. After opening the autoclave the HCl vapors were blown out with a stream of cold air (5 min) and the HPTLC plate was cooled to room temperature. In order to intensify and stabilize the fluorescence the plate was dipped in a solution of liquid paraffin in chloroform (30 + 70) for 15 s.

The digitalis glycosides yielded blue fluorescent zones in long- wavelength UV light ($\lambda = 365$ nm) (Fig. 1).

In situ quantitation: The fluorimetric analysis was carried out in UV light ($\lambda_{\text{exc}} = 365$ nm, $\lambda_{\text{fl}} > 430$ nm). The detection limit for digoxin was 300 pg per zone (Fig. 2).

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Hydrogen Peroxide Reagent

Reagent for:

- | | |
|----------------------|------------------------|
| ● Aromatic acids [1] | H_2O_2 |
| ● Thiabendazole [2] | $M_r = 34.01$ |

Preparation of Reagent

- | | |
|-------------------------|----------------------------------------------------------------------------------------------|
| Dipping solution | <i>Acids:</i> Make up 1 ml 30% hydrogen peroxide (Perhydrol®) to 100 ml with water [1]. |
| Spray solution | <i>Thiabendazole:</i> Make up 3 ml 30% hydrogen peroxide to 100 ml with 10% acetic acid [2]. |
| Storage | The reagent solutions should always be freshly made up. |
| Substances | Perhydrol® 30% H_2O_2
Acetic acid |

Reaction

Many carboxylic acids are converted into fluorescent derivatives by oxidation and UV irradiation. The reaction mechanism has not been elucidated.

Method

The chromatograms are freed from mobile phase, immersed for 3 s in the reagent solution or homogeneously sprayed with it and then subjected to intense UV

radiation ($\lambda = 365$ nm) for 30 s to 3 min while still moist [3]. Generally, after a few minutes, aromatic acids produce blue fluorescent chromatogram zones under long-wavelength UV light ($\lambda = 365$ nm) [3] which are not detectable in visible light. Within a few minutes thiabendazole yields bright fluorescent zones on a dark background under long-wavelength UV light ($\lambda = 356$ nm) [2].

Note: The dipping solution can also be sprayed on. The detection of the aromatic acids is best performed on cellulose layers, if ammonia-containing mobile phases have been employed. The reagent can also be employed on silica gel, aluminium oxide, RP 18 and polyamide layers.

Procedure Tested

Organic Acids [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	SIL G-25 UV ₂₅₄ plates (MACHEREY-NAGEL).
Mobile phase	Diisopropyl ether — formic acid — water (90 + 7 + 3).
Migration distance	10 cm
Running time	30 min

Detection and result: The chromatogram was dried in a stream of warm air for 10 min, immersed in the reagent solution for 3 s and then subjected to intense UV radiation (high pressure lamp, $\lambda = 365$ nm) for up to 10 min. Terephthalic (hR_f 0–5), pimelic (hR_f 55), suberic (hR_f 60), sebacic (hR_f 65–70) and benzoic acids (hR_f 70–75) together with sorbic, malic, adipic, citric, tartaric, lactic and fumaric acids only exhibited a reaction on silica gel layers at higher concentrations. 4-Hydroxybenzoic, salicylic and acetylsalicylic acids fluoresced light blue after irradiation. The detection limit per chromatogram zone was 0.5 μ g for salicylic acid and more than 5 μ g for benzoic acid.

In situ quantitation: The reagent had no advantages for the direct determination of the acids investigated; the determination of the intrinsic absorption or intrinsic fluorescences was to be preferred.

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8-Hydroxyquinoline Reagent

Reagent for

- Cations [1–10]
- 1,4-Benzodiazepines [11]



Preparation of the Reagent

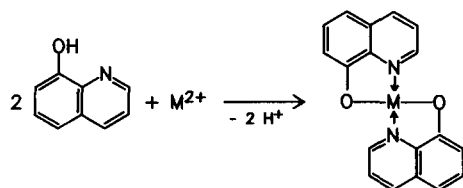
Dipping solution Dissolve 0.5 g 8-hydroxyquinoline in 100 ml ethyl acetate.

Storage The solution may be kept for several days.

Substances 8-Hydroxyquinoline
Ethyl acetate
Ethanol
Ammonia solution (25%)

Reaction

8-Hydroxyquinoline forms colored and fluorescent complexes with numerous metal cations.



Method

The chromatograms are dried in a stream of warm air, immersed in the reagent solution for 5 s, dried in the air and then placed for 5 min in a twin-trough chamber whose second trough contains 5 ml 25% ammonia solution. Directly after it is removed the plate is viewed under UV light ($\lambda = 254$ nm or 365 nm). The zones are mainly yellow in color; the colors of their fluorescence are listed in Table 1.

The dipping solution can also be employed as spray reagent, in this event 60% [6], 80% [5] or 95% ethanol [9] have been recommended in the literature. The reagent can be employed on silica gel, Dowex 50X4 (Na^+), cellulose and starch layers. The detection limit for 1,4-benzodiazapines is 100 ng per chromatogram zone [11].

Table 1: Colors of the 8-hydroxyquinoline complexes

Ion	Color		Detection limit [ng]
	Daylight	Fluorescence ($\lambda_{exc} = 365$ nm)	
Be^{2+}	green-yellow	yellow	200
Mg^{2+}	green-yellow	yellow	100
Ca^{2+}	yellow	yellow	500
Sr^{2+}	yellow	green-blue	200
Ba^{2+}	yellow	blue	500
$Sn^{2+/4+}$	yellow	*	*
$Cr^{3+/6+}$	black	*	*
$Fe^{2+/3+}$	black	purple	*
Al^{3+}	yellow	yellow	*
Ni^{3+}	yellow	red	*
Co^{2+}	yellow	red	*
Cu^{2+}	yellow	red	*
Bi^{2+}	yellow	red	*
Zn^{2+}	yellow	yellow	*
Cd^{2+}	yellow	yellow	*
Hg^{2+}	yellow	red	*

* = not reported

Procedure Tested

Alkaline Earths [4, 12]

Method	Ascending, one-dimensional development in a HPTLC chamber with chamber saturation.
Layer	HPTLC plates Cellulose (MERCK).
Mobile phase	Methanol – hydrochloric acid (25%) (80 + 20).
Migration distance	5 cm
Running time	20 min

Detection and result: The chromatogram was dried in a stream of warm air for 15 min, immersed in the reagent solution for 5 s, then dried in the air and finally placed in an ammonia chamber for 5 min. The plate was inspected under long-wavelength UV light ($\lambda = 365$ nm) immediately after removal from the chamber. The separation (Fig. 1) corresponded to the order of the group in the periodic table: Be^{2+} (hR_f 95–98), Mg^{2+} (hR_f 75–80), Ca^{2+} (hR_f 50–55), Sr^{2+} (hR_f 30–35, slight tailing) Ba^{2+} (hR_f 15–20).

The proportion of hydrochloric acid in the mobile phase was not to exceed 20%, so that complex formation did not occur and zone structure was not adversely affected. An excess of accompanying alkaline earth metal ions did not interfere with the separation but alkali metal cations did. The lithium cation fluoresced blue and lay at the same height as the magnesium cation, ammonium ions interfered with the calcium zone.

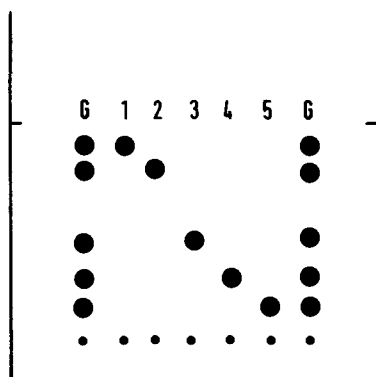


Fig. 1: Chromatographic separation of the alkaline earth cations. Mixture (G), Be^{2+} (1), Mg^{2+} (2), Ca^{2+} (3), Sr^{2+} (4), Ba^{2+} (5).

References

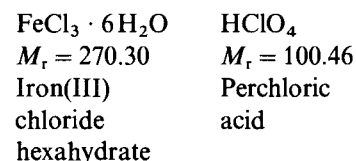
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Iron(III) Chloride – Perchloric Acid Reagent (FCPA Reagent)

Reagent for:

- Indole alkaloids [1 – 10]
e.g. from *rauwolfia* [1]
tabernaemontana [2, 7, 9]
mitragyna [3, 4]
strychnos [5]
synclisia [6]
cinchona [8]

- Indoles



Preparation of the Reagent

Solution I	Mix 2 ml perchloric acid (70%) carefully into 100 ml ethanol.
Solution II	Dissolve 1.35 g iron(III) chloride hexahydrate in 100 ml ethanol.
Dipping solution	Mix 100 ml solution I with 2 ml solution II [10].
Spray solution	Dissolve 5 g iron(III) chloride hexahydrate in a mixture of 50 ml water and 50 ml perchloric acid (70%).
Storage	The dipping solution may be stored for at least a week.
Substances	Iron(III) chloride hexahydrate Perchloric acid (70%) Ethanol

Reaction

The reagent mechanism has not yet been elucidated.

Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed for 4 s in the dipping solution or sprayed evenly with the spray solution until just transparent, dried briefly in the air and then heated to 110–120°C for 20–60 min.

Variously colored chromatogram zones are produced on a colorless background. Some of the zones appear before heating; their hues, which are to a great extent structure-specific, change during the heating process and they not infrequently fluoresce under short or long-wavelength UV light ($\lambda = 254$ or 365 nm).

Note: Indoles, that are substituted with oxygen in position 2 or 3, do not react [11]. The reagent can be employed on silica gel, kieselguhr and Si 50 000 layers. Aluminium oxide layers are not suitable [3].

Danger warning: Mists of perchloric acid can condense in the exhausts of fume cupboards and lead to uncontrolled explosions! So dipping is to be preferred.

Procedure Tested

Strychnine, Brucine [10]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK). The layers were pre-washed once with chloroform – methanol (1 + 1) and dried at 110°C for 10 min before application of samples.
Mobile phase	Acetone – toluene – 25% ammonia solution (40 + 15 + 5).

Migration distance 5 cm

Running time 10 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air (45 min), immersed in the dipping solution for 4 s, dried briefly in the air and heated to 110°C for 20 min.

Strychnine (hR_f 50–55) appeared as a red and brucine (hR_f 35) as a yellow chromatogram zone on a colorless background. The detection limit for both substances was 10 ng per chromatogram zone.

In situ quantitation: The light absorption in reflectance was measured at a wavelength $\lambda = 450$ nm (Fig. 1).

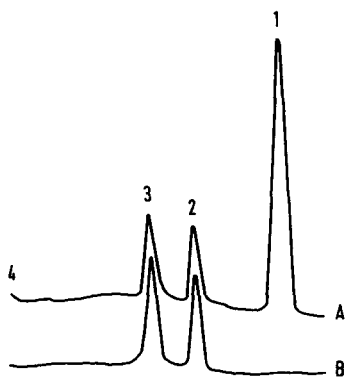


Fig. 1: Reflectance scan of a *nux vomica* extract (A) and a reference chromatogram containing 1 µg each of strychnine and brucine (B). Start (1), brucine (2), strychnine (3), front (4).

References

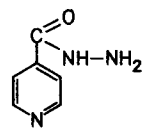
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Isonicotinic Acid Hydrazide Reagent (INH Reagent)

Reagent for:

- Δ^4 -3-Ketosteroids [1–6]
e.g. corticosteroids, androgens, gestagens



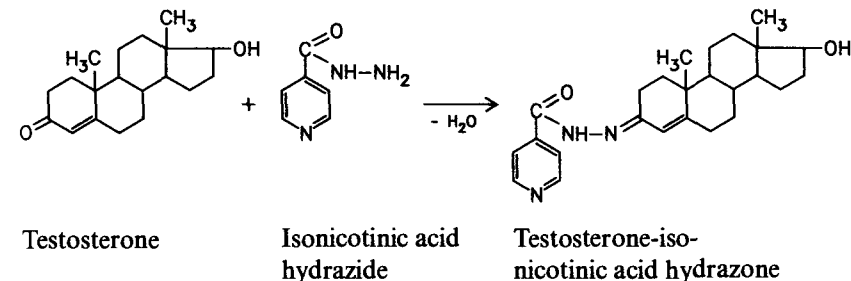
$C_6H_7N_3O$
 $M_r = 137.14$

Preparation of the Reagent

Dipping solution	Dissolve 1 g isonicotinic acid hydrazide (4-pyridinecarboxylic acid hydrazide, isoniazide) in 100 ml ethanol and add 500 μ l trifluoroacetic acid [1] or 1 ml glacial acetic acid.
Spray solution	Dissolve 0.8 g isonicotinic acid hydrazide in 200 ml methanol and add 1 ml hydrochloric acid (25%) [6] or glacial acetic acid [2].
Storage	Both solutions may be kept in the refrigerator for one week at 4°C.
Substances	4-Pyridinecarboxylic acid hydrazide Hydrochloric acid (25%) Trifluoroacetic acid Ethanol Methanol

Reaction

Isonicotinic acid hydrazide forms fluorescent hydrazones with ketosteroids.



Method

The chromatograms are dried in a stream of cold air, immersed in the dipping solution for 20 s or sprayed evenly with spraying solution, then dried in the air and left for a few minutes at room temperature.

The layer can then be dipped into liquid paraffin – *n*-hexane (1 + 2) to intensify the fluorescence [1].

Colored hydrazones are formed which fluoresce on a dark background in long-wavelength UV light ($\lambda = 365$ nm).

Note: Δ^4 -, $\Delta^{4,6}$ -, $\Delta^{1,4}$ - and Δ^1 -3-Ketosteroids react at different rates – as a function amongst other things of the acid strength of the reagent – so they can be differentiated [3, 5, 6].

The dipping solution may also be used as a spray solution. If this is done the reaction occurs more rapidly than reported in the literature for alcoholic spray solutions.

Silica gel, kieselguhr, Si 50000 and cellulose layers, amongst others, can be used as stationary phases.

Procedure Tested

Testosterone [1]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK). The layer was prewashed by developing once in chloroform and then twice in toluene – 2-propanol (10 + 1), with drying at 110°C for 30 min after each step.
Mobile phase	Toluene – 2-propanol (10 + 1).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatograms were freed from mobile phase (stream of cold air), immersed in the reagent solution for 20 s, then dried in the air and finally kept at room temperature for 20 min. Testosterone (hR_f 35–40) fluoresced pale blue in long-wavelength UV light ($\lambda = 365$ nm, Fig. 1).

In situ quantitation: The chromatogram was then dipped into liquid paraffin – *n*-hexane (10 + 20) to increase the intensity of the fluorescence by a factor of ten and to stabilize it. The detection limit for testosterone is less than 500 pg per chromatogram zone ($\lambda_{exc} = 365$ nm; $\lambda_{fl} > 430$ nm).

The separation of a mixture of Δ^4 -3-ketosteroids is illustrated in Figure 2.

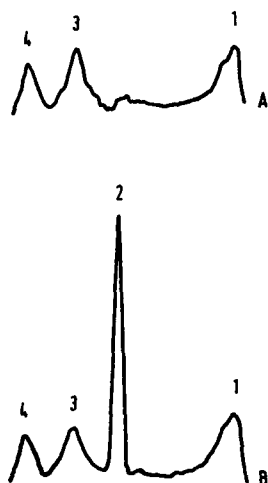


Fig. 1: Fluorescence scan of a blank track (A) and of the testosterone derivative (B, 2 µg). Start (1), testosterone-INH derivative (hR_f 39) (2), unknown substances (3, 4).

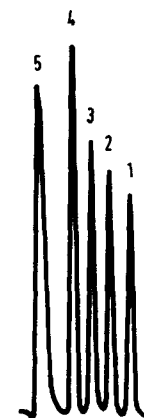


Fig. 2: Fluorescence scan of a Δ^4 -3-ketosteroid mixture after INH treatment. Aldosterone (1), corticosterone (2), cortisone (3), testosterone (4), progesterone (5).

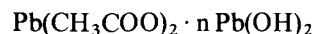
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Lead(II) Acetate Basic Reagent

Reagent for:

- Flavonoids [1–3]
- Aryl-substituted thiourea derivatives (thiocarbamide derivatives) [4]
- Oligogalactoside uronic acids [5]

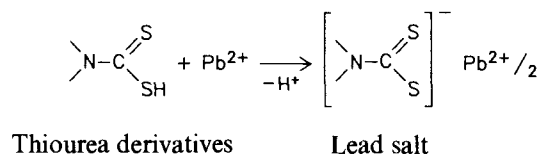


Preparation of Reagent

Dipping solution	Basic lead(II) acetate solution (lead content: 17.5–19%).
Storage	The reagent solution may be stored over a longer period of time, providing it is tightly stoppered.
Substances	Lead acetate (17.5–19% Pb) (lead(II) acetate solution, basic)

Reaction

Lead(II) acetate yields colored lead salts with flavonoids and thiourea derivatives.



Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the reagent solution for 1 s or homogeneously sprayed with it until the layer starts to be transparent and heated to 50–80 °C for 5–20 min.

Chromatogram zones of various colors sometimes appear even before heating, these fluoresce under long-wavelength UV light ($\lambda = 365 \text{ nm}$) in the case of some substances.

Note: The detection limits per chromatogram zone are ca. 1 μg substance in the case of aryl-substituted thioureas [4], but even at 50 μg per zone diallate and triallate did not produce any reaction [6]. The reagent should be employed undiluted (cf. "Procedure Tested", Fig. 2).

The reagent can be employed on silica gel, kieselguhr, Si 50 000, cellulose and polyamide layers.

Procedure Tested

Flavone Glucosides [7]

Method	Ascending, one-dimensional development in a HPTLC trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Ethyl acetate – dichloromethane – formic acid – water (35 + 15 + 5 + 3).
Migration distance	5 cm
Running time	13 min

Detection and result: The chromatogram was freed from mobile phase, immersed in the reagent solution for 1 s and then heated to 80 °C for 10 min. Rutin (hR_f 5–10), kaempferol glucoside (hR_f 10–15), hyperoside (hR_f 20–25), isoquercitrin (hR_f 25–30), quercitrin (hR_f 40–45), luteolin (hR_f 75–80), quercetin (hR_f 80–85) and isorhamnetin (hR_f 80–85) yielded yellow to brown chromatogram zones on a pale background; under long-wavelength UV light ($\lambda = 365 \text{ nm}$) these pro-

duced yellow to orange fluorescence. The detection limits were 50–100 ng substance per chromatogram zone (Fig. 1). The influence of the reagent concentration on the sensitivity of detection is illustrated in Figure 2.

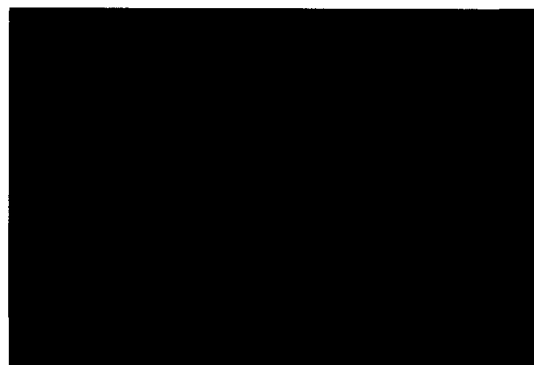


Fig. 1

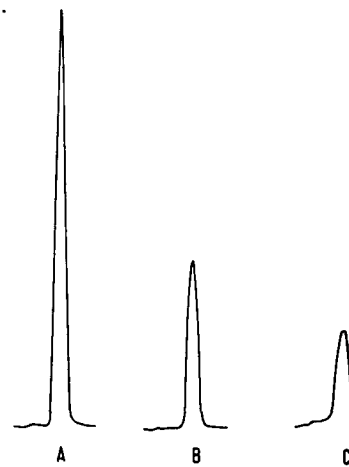


Fig. 2

Fig. 1: Chromatographic separation of flavone glucosides

Fig. 2: The influence of the reagent concentration on the sensitivity of detection; detection of equal amounts of luteolin with basic lead acetate solution that was (A) undiluted, (B) diluted 1 + 4 and (C) diluted 1 + 50.

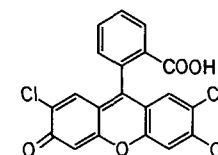
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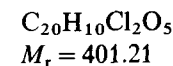
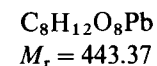
Lead(IV) Acetate – Dichlorofluorescein Reagent

Reagent for:

- Vicinal diols [1–5]
e.g. sugar acids [1, 2]
monosaccharides [2–4]
oligosaccharides [2, 3]
sugar alcohols [2–4]
cyclitols [5]
glycerol [2, 3]



- Glycosides
e.g. menthyl glucoside [6]
arbutin [7]



- Phenols [8]

Lead(IV) acetate

Dichloro-
fluorescein

Preparation of the Reagent

- Solution I** Two percent to saturated solution of lead(IV) acetate in glacial acetic acid.
- Solution II** Dissolve 0.2 to 1 g 2',7'-dichlorofluorescein in 100 ml ethanol.
- Dipping solution** Mix 5 ml solution I and 5 ml solution II and make up to 200 ml with toluene immediately before use.
- Storage** Solutions I and II on their own are stable for several days, the dipping solution should always be freshly made up before use.

Substances	Lead(IV) acetate
	2',7'-Dichlorofluorescein
	Acetic acid (glacial acetic acid) 100%
	Ethanol
	Toluene

Reaction

The reaction is based, on the one hand, on the oxidative cleavage of vicinal diols by lead(IV) acetate and, on the other hand, on the reaction of dichlorofluorescein with lead(IV) acetate to yield a nonfluorescent oxidation product. The dichlorofluorescein only maintains its fluorescence in the chromatogram zones where the lead(IV) acetate has been consumed by the glycol cleavage reaction [1].

Method

The chromatogram is freed from mobile phase, immersed in the reagent solution for 8–10 s, dried briefly in a stream of warm air and heated in the drying cupboard to 100°C for 3–30 min.

Chromatogram zones are formed that exhibit yellow fluorescence in shortwave UV light ($\lambda = 254$ nm); they are sometimes also recognizable as orange-red spots in visible light.

Note: The full fluorescence intensity usually only develops about 30 min after the dipping process; it then remains stable for several days if the chromatograms are stored in the dark [1, 5]. Fluorescein sodium can be employed in the reagent in place of 2',7'-dichlorofluorescein [5]. The detection limits lie in the lower nanogram to picogram range [1, 5].

The reagent can be employed on silica gel, Si 50 000 and kieselguhr layers.

Remark: All substances with vicinal diol groups (sugars, sugar alcohols, glycosides etc.) yield a yellow-green fluorescence with this reagent. In order to determine which zones are produced by sugars the plate can be sprayed later with naphthalene-1,3-diol — sulfuric acid reagent which colors the sugars but not the sugar alcohols [4, 9].

Procedure Tested

Mono- and Sesquiterpene Glucosides [6], Arbutin and Methylarbutin [7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	<i>Terpene glucosides</i> : chloroform — methanol (80 + 20). <i>Arbutin</i> : ethyl acetate — methanol — water (100 + 17 + 14).
Migration distance	5 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase, immersed in the reagent solution for 1 s, dried briefly in a stream of warm air and heated to 100°C for 5 min.

The glucosides of menthol, citronellol, nerol, geraniol, *cis*-myrtenol, L-borneol, linalool and α -terpineol yielded yellow-green fluorescent chromatogram zones in long-wavelength UV light ($\lambda = 365$ nm). The same applied to arbutin (hR_f 45–50).

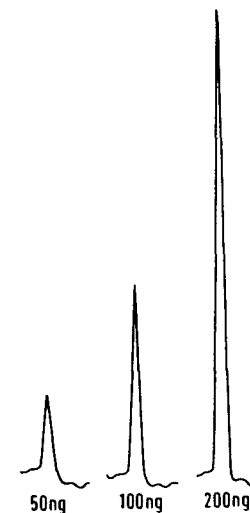


Fig. 1: Fluorescence scan of chromatogram tracks with 50, 100 and 200 ng arbutin per chromatogram zone.

In situ quantitation: The fluorimetric analysis of monoterpene glucosides could be performed with advantage at $\lambda_{exc} = 313 \text{ nm}$ and $\lambda_{fl} > 390 \text{ nm}$. The detection limits of arbutin and L-menthylglucoside were 1–5 ng and 15 ng substance per chromatogram respectively (Fig. 1).

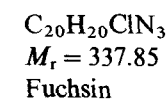
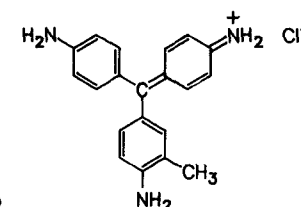
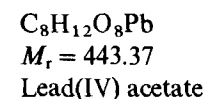
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Lead(IV) Acetate – Fuchsin Reagent

Reagent for:

- α -Diol groupings
e.g. sugar alcohols [1]
sugars [4]



Preparation of Reagent

Dipping solution I Dissolve 1 g lead(IV) acetate (lead tetraacetate) in 100 ml ethanol.

Dipping solution II Dissolve 10 mg fuchsin in 100 ml methanol.

Spray solution I Dissolve 1 g lead(IV) acetate in 100 ml toluene.

Spray solution II Dissolve 50 mg fuchsin in 100 ml methanol.

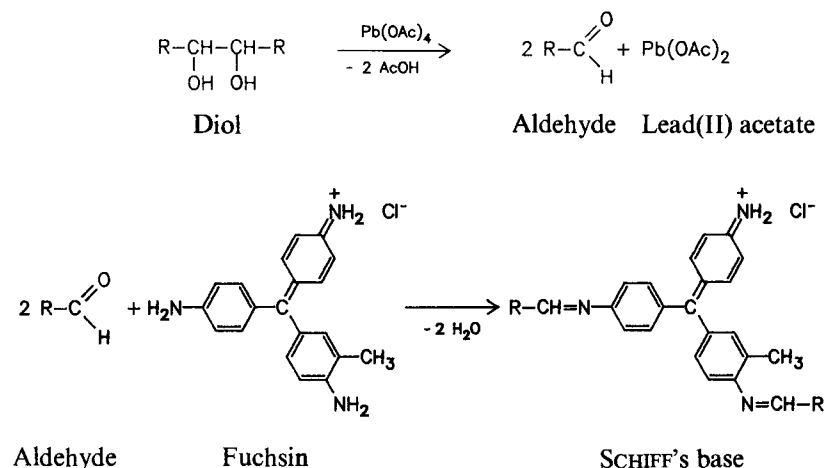
Storage Solution I should always be freshly prepared, solution II may be stored for a longer period of time.

Substances

- Lead(IV) acetate
- Fuchsin
- Toluene
- Methanol
- Ethanol

Reaction

The reaction depends, on the one hand, on the fact that fuchsin is decolorized by oxidizing agents (e.g. lead(IV) acetate) and, on the other hand, on the fact that lead(IV) acetate is reduced by compounds containing α -diol groups. It is, therefore, no longer available to decolorize the fuchsin. The fuchsin undergoes a SCHIFF reaction with the aldehydes that are formed [2].



Method

The chromatogram is freed from mobile phase, immersed for 3 s in dipping solution I or sprayed homogeneously with spray solution I and then dried for ca. 10 min in a stream of cold air. It is then immersed for 1 s in dipping solution II or sprayed evenly with spray solution II.

Reaction usually occurs immediately or occasionally after heating briefly to 140°C, to yield red-violet chromatogram zones on a pale yellow-beige background.

Note: The color of the zones persists for a long period, but changes to blue-violet [1]. Rosaniline [1, 2] can be employed instead of fuchsin. With sugar alcohols lead(IV) acetate alone yields white zones on a brown background (detection limit 1–2 μg per chromatogram zone) [3].

The reagent can be employed on silica gel, kieselguhr and Si 50 000 layers.

Procedure Tested

Mono-, Di- and Trisaccharides [4]

Method	Ascending, one-dimensional double development in a trough chamber at 80°C without chamber saturation.
Layer	HPTLC plates Si 50000 (MERCK), which had been pre-washed before sample application by developing once with chloroform — methanol and then dried at 110°C for 30 min.
Mobile phase	Acetonitrile — water (85 + 15).
Migration distance	2 × 6 cm
Running time	2 × 8 min (5 min intermediate drying in stream of warm air)

Detection and result: The chromatogram was dried for 15 min in a stream of cold air, immersed in dipping solution I for 3 s and dried for 10 min in a stream of cold air. It was then immersed for 1 s in dipping solution II, dried briefly in a stream of warm air and heated to 140°C for 1 min (hot plate).

Raffinose (hR_f 5–10), lactose (hR_f 15–20), sucrose (hR_f 30–35), glucose (hR_f 45–50) and fructose (hR_f 60–65) yielded violet chromatogram zones with a weak,

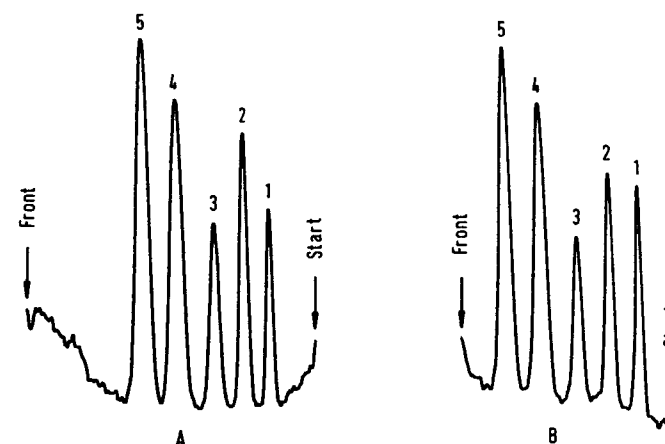


Fig. 1: Absorption scan (A) and fluorescence scan (B) of a chromatogram track with 200 ng sugar per chromatogram zone: raffinose (1), lactose (2), sucrose (3), glucose (4) and fructose (5).

pale blue fluorescence on a pale beige background. The detection limits were 20 ng substance per chromatogram zone. When the chromatogram was subsequently treated with hydrochloric acid vapor for 15 min (twin-trough chamber, 32% hydrochloric acid in the unoccupied half) the zones became more intensely violet-colored against a pale violet background; under long-wavelength UV light ($\lambda = 365$ nm) the zones fluoresced red against a dark background. Here too the detection limits were 20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric determination in reflectance was performed at $\lambda = 560$ nm, the fluorimetric analysis was performed at $\lambda_{\text{exc}} = 436$ nm and $\lambda_{\text{fl}} > 560$ nm (Fig. 1).

References

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Manganese(II) Chloride – Sulfuric Acid Reagent

Reagent for:

● Cholesterol, cholesteryl esters [1–4]		
● Phospholipids, lipids [1, 5]		
● Triglycerides, free fatty acids [1]	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	H_2SO_4
● Bile acids [2]	$M_r = 197.91$	$M_r = 98.08$
● Ketosteroids [3]	Manganese(II) chloride	Sulfuric acid
● Estrogens [6]	tetrahydrate	

Preparation of Reagent

Dipping solution	Dissolve 0.2 g manganese chloride tetrahydrate in 30 ml water and add 30 ml methanol. Mix well and then carefully add 2 ml conc. sulfuric acid dropwise.
Storage	The solution should always be freshly made up for quantitative evaluations.
Substances	Manganese(II) chloride tetrahydrate Methanol Sulfuric acid (95–97%)

Reaction

The reaction mechanism has not yet been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in the reagent solution for 1 s and then heated to 100–120°C for 10–15 min. Colored zones appear on a colorless background [2] (Table 1) and these fluoresce pale blue in long-wavelength UV light ($\lambda = 365$ nm) [1].

Table 1: Colors of the chromatogram zones

Substance	Color
Cholesterol	pink
Cholic acid	deep yellow
Chenodesoxycholic acid	grey-green
Desoxycholic acid	yellow
Hydodesoxycholic acid	brown
Lithocholic acid	pale pink

The pink color of the cholesterol begins to fade after 5 min while the color of the bile acids deepens [2]. The visual detection limit in visible light is 1 μ g for cholesterol and 2 μ g per chromatogram zone for bile acids [2]. Fluorimetric detection is more sensitive by a factor of 1000!

Note: The reagent, which may also be used as spray solution, can be applied to silica gel, RP-2, RP-8, RP-18 and CN layers.

Procedure Tested

Cholesterol, Coprostanone, Coprostanol, 4-Cholesten-3-one, 5 α -Cholestan-3-one [3]

Method Ascending, one-dimensional development in a trough chamber. The HPTLC plates were preconditioned for 30 min at 0% rel. humidity (over conc. sulfuric acid) after sample application and then developed immediately.

Layer HPTLC plates Silica gel 60 F₂₅₄ (MERCK), which had been prewashed by developing once up to the edge of the plate with chloroform — methanol (1 + 1) and then activated to 110°C for 30 min.

Mobile phase Cyclohexane — diethyl ether (1 + 1).

Migration distance 6 cm

Running time ca. 15 min

Detection and result: The developed chromatogram was dried in a stream of cold air, immersed in the reagent solution for 1 s and then heated to 120°C for 15 min.

The result was that light blue fluorescent zones were visible under long-wavelength UV light ($\lambda = 365$ nm). Before fluorimetric analysis the chromatogram was dipped for 1 s into liquid paraffin — *n*-hexane (1 + 2) to enhance (by a factor of 2 to 8) and stabilize the intensity of the fluorescence and then dried for 1 min in a stream of cold air. The quantitation ($\lambda_{\text{exc}} = 365$ nm; $\lambda_{\text{fl}} > 430$ nm) was carried out after 1 h since it was only then that the fluorescence intensity had stabilized.

Note: Coprostanone, 4-cholesten-3-one and 5 α -cholestan-3-one could be detected more sensitively, if the dried chromatogram was irradiated with intense long-wavelength UV light ($\lambda = 365$ nm) for 2 min before being immersed in the reagent solution. Figure 1 illustrates a separation of these substances. The detection limits

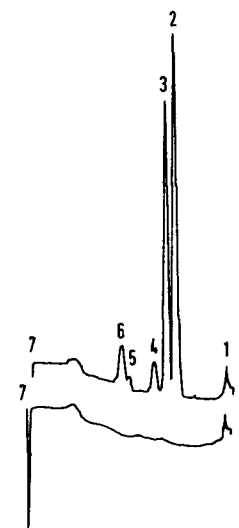


Fig. 1: Fluorescence scans of a blank (A) and of a mixture (B) of cholesterol (2), coprostanol (3), 4-cholesten-3-one (4), 5 α -cholestan-3-one (5) and coprostanone (6), start (1), solvent front (7).

were 1 ng per chromatogram zone for cholesterol (hR_f 20–25) and coprostanol (hR_f 25–30) and 50 ng per chromatogram zone for 4-cholesten-3-one (hR_f 40–45), 5 α -cholestan-3-one (hR_f 60–65) and coprostanone (hR_f 70–75).

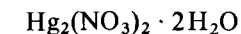
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Mercury(I) Nitrate Reagent

Reagent for:

- Barbiturates and barbiturate metabolites [1–4]
- Various pharmaceuticals
e.g. pentenamide [1]
gluthethimide, primidone, phenytoin [2]
- Organophosphorus insecticides [5]
- Succinimides [6]
- Thiourea [7]



$$M_r = 561.22$$

Preparation of Reagent

- | | |
|-----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Spray solution | Grind 5 g mercury(I) nitrate dihydrate with 100 ml water in a mortar and transfer it to a storage vessel along with the sediment; the supernatant is fit for use as long as the sediment remains white [1]. |
| Storage | The reagent may be stored for an extended period; new reagent must be made up when the sediment becomes grey or yellow in color [1]. |
| Substances | Mercury(I) nitrate dihydrate |

Reaction

The reaction mechanism has not yet been elucidated.

Method

The chromatograms are dried in a stream of warm air or at 105°C for 10 min, cooled to room temperature and sprayed homogeneously with the spray reagent until they start to become transparent.

Grey-black chromatogram zones are produced on a white background, usually appearing immediately but sometimes only after a few minutes. In the case of insecticides the chromatograms are heated after spraying [5].

Note: Traces of ammonia from the mobile phase should be removed from the plate completely to avoid background discoloration (grey veil) [1]. If the layer is sprayed too heavily the initially grey-black chromatogram zones can fade again [2]. The reagent which is usually employed as a 1 to 2% solution [2, 3, 6, 7] can be treated with a few drops of nitric acid to clarify the solution [2].

Barbiturate metabolites are more heavily colored by the mercury(I) nitrate reagent (exception: allobarbitol), while unaltered barbiturates react more sensitively to the mercury(II)-diphenylcarbazone reagent (q.v.) [1].

The reagent can be employed on silica gel, kieselguhr, aluminium oxide and cellulose layers.

Procedure Tested

Barbiturates and Metabolites [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Chloroform – acetone (80 + 20).
Migration distance	ca. 6 cm
Running time	7 min

Detection and result: The developed chromatogram was dried for 5 min in a stream of hot air (120–150°C) then treated with a stream of cold air for 5 min. It was

then sprayed with reagent solution until the layer began to be transparent. After 5 min barbiturates and their metabolites produced grey to black zones on a colorless background (Fig. 1).

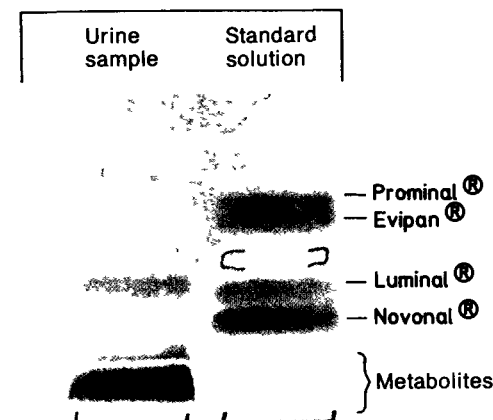


Fig. 1: Chromatograms of a urine sample and a standard solution.

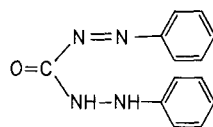
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Mercury(II) Salt – Diphenylcarbazone Reagent

Reagent for:

- Barbiturates, barbiturate metabolites [1 – 6]
- Sulfonamides [2]
- Various pharmaceuticals
e.g. glutethimide [2 – 4]
lysergic acid [2]
primidone, phenytoin [3]
- Organophosphorus insecticides [7]



HgCl_2
 $M_r = 271.50$
Mercury(II)
chloride

$\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$
 $M_r = 342.62$
Mercury(II) nitrate
monohydrate

$\text{C}_{13}\text{H}_{12}\text{N}_4\text{O}$
 $M_r = 240.27$
Diphenylcarbazone

Preparation of Reagent

- Solution I** Dissolve 100 mg 1,5-diphenylcarbazone in 100 ml ethanol (96%).
- Solution II** Dissolve 40 g ammonium acetate in 100 ml water and adjust to pH 3.5 with ca. 30 ml nitric acid (65%).
- Spray solution Ia** Dissolve 2 g mercury(II) chloride in 100 ml ethanol [1, 3, 4].

- Spray solution Ib** Dissolve 200 mg 1,5-diphenylcarbazone in 100 ml ethanol [1, 3, 4].
- Spray solution IIa** Dissolve 200 mg mercury(II) nitrate monohydrate in 100 ml nitric acid ($c = 0.1 \text{ mol/l}$) [6].
- Spray solution IIb** Mix 4 ml solution I and 26 ml solution II [6].
- Storage** Solutions I and II and spray solutions Ia and Ib may be stored in the refrigerator for ca. 1 month, both spray solutions IIa and IIb may be kept for ca. 1 week.
- Substances** 1,5-Diphenylcarbazone
Mercury(II) nitrate monohydrate
Mercury(II) chloride
Nitric acid 0.1 mol/l Titrisol
Nitric acid (65%)
Ethanol
Ammonium acetate

Reaction

The reaction mechanism has not yet been elucidated.

Method

Variant I: The chromatogram is dried in a stream of warm air or in the drying cupboard (10 min, 120 °C), cooled to room temperature and either sprayed with spray solutions Ia and Ib one after the other (with brief drying in a stream of cold air in between) [1] or sprayed with a mixture of equal volumes of these two spray reagents until the layer begins to be transparent [2, 4].

In the case of organophosphorus insecticides [7] and usually also in the case of barbiturates and other pharmaceuticals [1, 3] white chromatogram zones are produced on a lilac background. Barbiturates sometimes appear — especially after the use of basic mobile phases — as blue-violet colored zones on a pink background [2, 5].

Variant II: The chromatogram is dried in a stream of warm air or in the drying cupboard (10 min, 120°C), cooled to room temperature and then sprayed twice with spray solution IIa until the layer is transparent (dry in a stream of warm air after each spray step!). It is then sprayed with a small amount of spray solution IIb [6].

Barbiturates and their metabolites always appear as red-violet chromatogram zones on a white background with this variant of the reagent [6].

Note: The reaction for barbiturates according to variant I is increased in sensitivity if the chromatogram is exposed to direct sunlight or UV light after it has been sprayed; this causes the background coloration to fade almost completely and the blue zones stand out more distinctly [4].

While barbiturate metabolites are more intensely colored by the mercury(I) nitrate reagent (q.v.) the unaltered barbiturates react more sensitively to the mercury(II) diphenylcarbazone reagent; the detection limits lie between 0.05 µg (Luminal®) and 10 µg (Prominal®) per chromatogram zone [6].

The reagent can be employed on silica gel and kieselguhr layers.

Procedure Tested

Barbiturates and Metabolites [6]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Chloroform — acetone (80 + 20).
Migration distance	ca. 6 cm
Running time	7 min

Detection and result: The chromatogram was dried for 5 min in a stream of hot air (120–150°C) then treated with a stream of cold air for 5 min. It was then sprayed twice to transparency with spray solution IIa (drying in between for 3 min in a stream of hot air). It was then heated in a stream of hot air for 5 min. After cooling for 5 min in a stream of cold air it was sprayed with a small amount of spray

solution IIb. Barbiturates and their metabolites appeared as violet chromatogram zones on an almost colorless background. The detection limits for barbiturates lay between 50 ng (Luminal®) and 10 µg (Prominal®, Fig. 1).

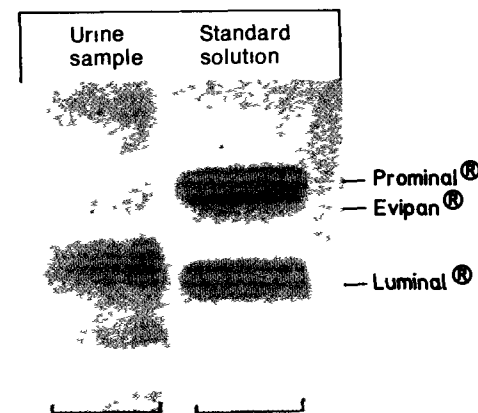


Fig. 1: Chromatograms of a urine sample and a standard solution.

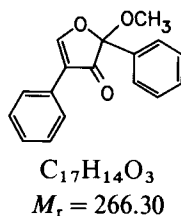
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2-Methoxy-2,4-diphenyl-3(2H)-furanone Reagent (MDPF Reagent)

Reagent for:

- Amines
e.g. colchicine [1]



Preparation of Reagent

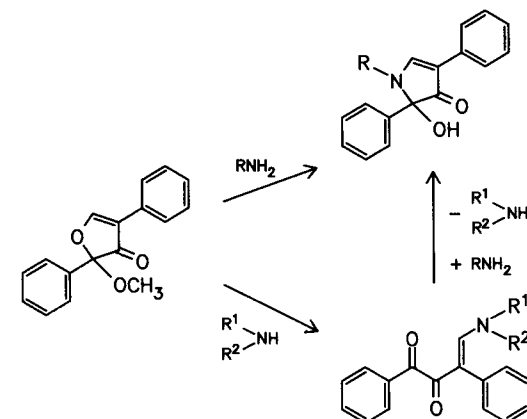
Dipping solution Dissolve 25 mg 2-methoxy-2,4-diphenyl-3(2H)-furanone in 50 ml methanol.

Storage The dipping solution may be stored for several days.

Substances 2-Methoxy-2,4-diphenyl-3(2H)-furanone
Methanol

Reaction

MDPF reacts directly with primary amines to form fluorescent products. Secondary amines yield nonfluorescent derivatives, which may be converted into fluorescent substances by a further reaction with primary amines [2].



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the reagent solution for 4 s or sprayed evenly with it and then heated to 110°C for 20 min.

Yellow chromatogram zones are formed on a colorless background; these exhibit yellow fluorescence in long-wavelength UV light ($\lambda = 365$ nm).

Note: The reagent can be applied to silica gel, Si 50000 and kieselguhr layers.

Procedure Tested

Colchicine [1]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer HPTLC plates Silica gel 60 (MERCK) which had been washed by developing once with chloroform – methanol (1 + 1) and then dried at 110°C for 30 min before applying the samples.

Mobile phase Acetone – toluene – ammonia solution (25%) (40 + 15 + 5).

Migration distance 5 cm

Running time 10 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air (45 min), immersed in the reagent solution for 4 s and then heated to 110 °C for 20 min.

Colchicine (R_f 35–40) appeared as yellow fluorescent zone on a dark background in long-wavelength UV light ($\lambda = 365$ nm). The detection limit was 10 ng per chromatogram zone.

In situ quantitation: The fluorimetric analysis was carried out with excitation at $\lambda_{exc} = 313$ nm and evaluation at $\lambda_{fl} > 390$ nm (Fig. 1).

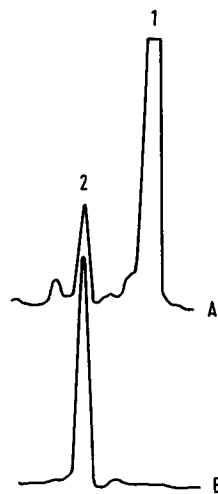


Fig. 1: Fluorescence scan of a chromatogram track with an extract of *Colchicum autumnale* (A) and of a reference track (B, 1 µg colchicine); start (1), colchicine (2).

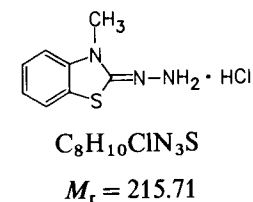
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3-Methyl-2-benzothiazolinone-hydrazone Reagent (MBTH Reagent, BESTHORN's Reagent)

Reagent for:

- Carbonyl compounds [1]
- Mycotoxins
 e.g. patulin [2–10]
 moniliformine [11]
- Penicillic acid [12]



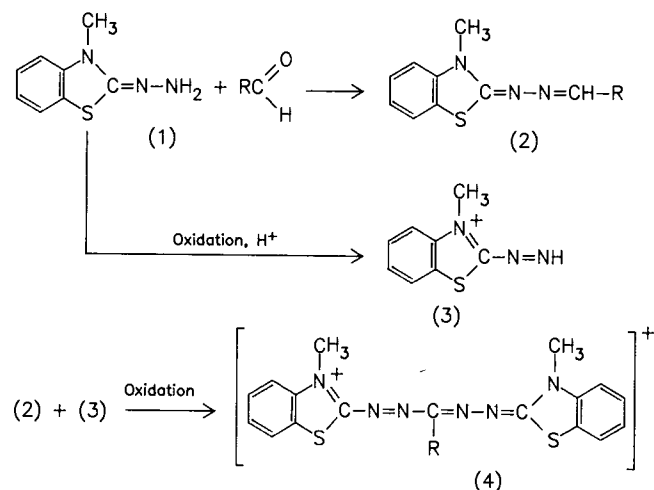
Preparation of Reagent

Dipping solution Dissolve 0.5–1 g 3-methyl-2-benzothiazolinone-hydrazone hydrochloride (BESTHORN's hydrazone) in 100 ml methanol – water (1 + 1), water [2, 4–8] or methanol [3, 11]. If precipitation occurs filter the solution before use.

Storage The dipping solution is only stable for a few days and, hence, should be freshly made up each time.

Substances 3-Methyl-2-benzothiazolinone-hydrazone hydrochloride
 Methanol

Reaction (according to [13])



Method

The chromatogram is freed from mobile phase, immersed in the reagent solution for 1 s or homogeneously sprayed with it and then heated to 110–130°C for up to 2 h. The chromatographic zones produced are usually blue-violet (e.g. patulin, moniliformine) on a pale background; they exhibit yellow to yellow-orange fluorescence (penicillic acid, patulin) in long-wavelength UV light ($\lambda = 365$ nm).

Note: The reagent can be employed on silica gel, kieselguhr, cellulose and polyamide layers. When left exposed to air the whole chromatogram is slowly colored blue because of the formation of the blue cation (3) (see Reaction). The detection limits for patulin, moniliformine and penicillic acid are ca. 50 ng per chromatogram zone.

Procedure Tested

Penicillic Acid [14]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene – ethyl acetate – formic acid (60 + 30 + 10).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was freed from mobile phase, immersed in reagent solution for 1 s and then heated to 130°C for 90–120 min. Penicillic acid (R_f 45–50) yielded yellow chromatogram zones which fluoresced yellow in long-wavelength UV light ($\lambda = 365$ nm). The detection limits were 50 to 100 ng per chromatogram zone.

In situ quantitation: Fluorimetric analysis was carried out at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 560$ nm (Fig. 1).

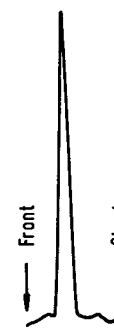


Fig. 1: Fluorescence scan of a chromatogram track of 0.5 μ g penicillic acid.

References

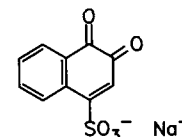
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1,2-Naphthoquinone-4-sulfonic Acid – Perchloric Acid – Formaldehyde Reagent

Reagent for:

● Sterols

e.g. ergosterol
stigmasterol
cholesterol [1]

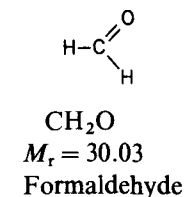


● Alkaloids

e.g. codeine
morphine
heroin
6-monoacetyl-
morphine [2]

$C_{10}H_5NaO_5S$
 $M_r = 260.20$
1,2-Naphtho-
quinone-4-
sulfonic acid
sodium salt

$HClO_4$
 $M_r = 100.46$
Perchloric
acid



Preparation of Reagent

- Dipping solution** Dissolve 100 mg 1,2-naphthoquinone-4-sulfonic acid sodium salt in 40 ml ethanol and add 20 ml perchloric acid (70%), 18 ml water and 2 ml formaldehyde solution in that order.
- Storage** The reagent solution may be kept for ca. 4 weeks in the refrigerator.
- Substances** 1,2-Naphthoquinone-4-sulfonic acid
sodium salt
Ethanol
Perchloric acid (70%)
Formaldehyde solution (37%)

Reaction

The reaction mechanism has not been elucidated. It is possible that formaldehyde reacts by oxidation as in the MARQUIS' reaction (see formaldehyde – sulfuric acid reagent), whereby colored salts are formed with naphthoquinone sulfonic acid.

Method

The chromatograms are freed from mobile phase (stream of warm air 5 min), immersed for 4 s in the reagent solution or sprayed homogeneously with it until they begin to be transparent and then heated to 70 °C for ca. 10 min.

Chromatogram zones are produced that are initially pink but turn blue as the heating continues; they are on a pale blue background.

Note: The color development depends on the temperature and duration of heating [1]. The detection limit for sterols and morphine alkaloids is in the lower nanogram range [1, 2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Morphine Alkaloids [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK) which had been prewashed once with chloroform – methanol (50 + 50) and then dried at 110 °C for 20 min.
Mobile phase	Methanol – chloroform – water (12 + 8 + 2).
Migration distance	6 cm
Running time	20 min

Detection and result: The chromatogram was dried in a stream of warm air for 5 min, immersed in the reagent solution for 4 s and heated to 70 °C for ca. 10 min.

Morphine (hR_f 25–30), codeine (hR_f 30–35), 6-monoacetylmorphine (hR_f 40–45) and heroin (hR_f 50–55) yielded blue chromatogram zones on a pale blue background. The detection limits were 10 to 20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric analysis was made in reflectance at $\lambda = 610$ nm (Fig. 1).

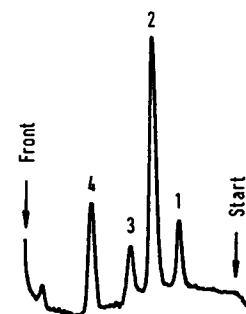


Fig. 1: Reflectance scan of the alkaloid mixture with ca. 50 ng substance per chromatogram zone. Morphine (1), codeine (2), 6-monoacetylmorphine (3), heroin (4).

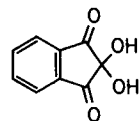
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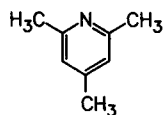
Ninhydrin – Collidine Reagent

Reagent for:

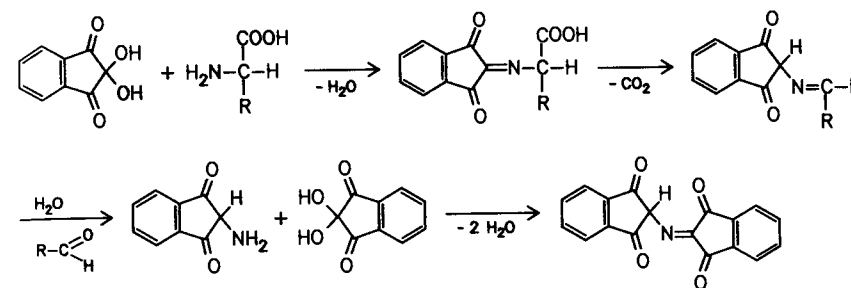
- Amines [1]
- Aminoglycoside antibiotics [2, 3]
- Amino acids [5 – 7]
- Peptides [6]



$C_9H_6O_4$
 $M_r = 178.15$
Ninhydrin



$C_8H_{11}N$
 $M_r = 121.18$
Collidine



Method

The chromatogram is freed from mobile phase, immersed for 1 s in the reagent solution and then heated to 95–120°C for 5–10 min. After 15 min stabilization time mainly reddish but sometimes blue substance zones appear on a pale background.

Note: The reagent can be employed on silica gel and cellulose layers and the dipping solution can also be employed as a spray solution. The addition of collidine

Preparation of Reagent

Dipping solution Dissolve 0.3 g ninhydrin (2,2-dihydroxy-1,3-indanedione) in 95 ml 2-propanol and add 5 ml collidine (2,4,6-trimethylpyridine) and 5 ml acetic acid (96%).

Storage If the solvents are not sufficiently pure the solution may only be kept for a short time.

Substances Ninhydrin
2,4,6-Trimethylpyridine
Acetic acid (96%)
2-Propanol

Reaction

The course of the reaction has not been fully explained; one possible route is:

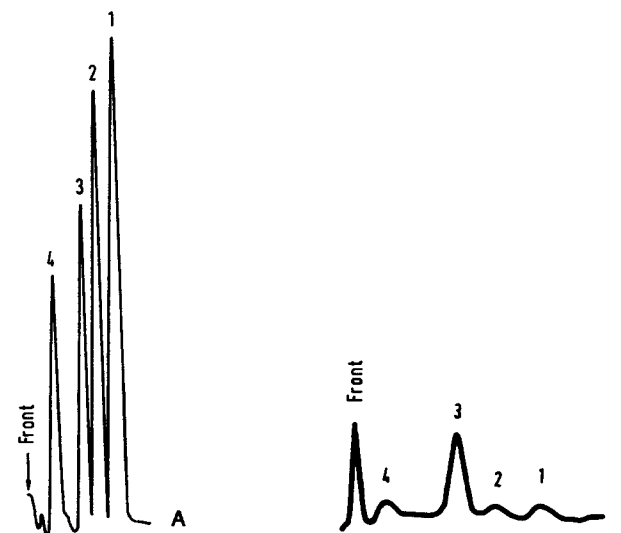


Fig. 1: Comparative recordings of the reflectance scans of a mixture of phenylethylamine (1), tyramine (2), serotonin (3) and histamine (4); A. ninhydrin reagent (q.v.), B. ninhydrin – collidine reagent.

buffers the layer and allows, for example, the differentiation of glycine and serine by color [4]. In the case of biogenic amines the reagent is much more sensitive if collidine is omitted* (Fig. 1). Ammonia vapors interfere with the reaction. If the zones fade rapidly they can be stabilized by complex formation by the addition of tin, copper [8], cobalt or cadmium salts to the reagent (cf. also copper(II) nitrate reagent for the stabilization of "ninhydrin spots").

Procedure Tested

Gentamycin Complex [2, 3, 10]

Method	Ascending, one-dimensional development at 10–12°C in a twin-trough chamber, with 25% ammonia in that part not containing mobile phase. The chamber was equilibrated for 15 min before starting development.
Layer	HPTLC plates Silica gel 60 (MERCK) prewashed by developing three times with chloroform — methanol (50 + 50) with intermediate and final drying at 110°C for 30 min.
Mobile phase	Chloroform — ethanol — ammonia solution (25%) (10 + 9 + 10). <i>The lower organic phase was employed.</i>
Migration distance	ca. 5 cm
Running time	ca. 20 min

Detection and result: The chromatogram was treated in a stream of cold air for 30 min in order to remove the mobile phase, it was then immersed in the reagent solution for 1 s and finally, after drying in the stream of warm air it was heated to 95°C for 10 min. After 15 min stabilization time at room temperature blue chromatogram zones are produced on a colorless background.

Note: Under these conditions gentamycins C₂ and C_{2a} form a common zone. A separation can be achieved, for example, with methanol — 0.1 mol/l LiCl in 32% aqueous ammonia solution (5 + 25) on KC18F plates (WHATMAN) [9].

* Jork, H.: Private communication, Universität des Saarlandes, D-6600 Saarbrücken, 1988

In situ quantitation: The direct quantitative analysis of the blue-violet derivatives should be made in reflectance at $\lambda = 580$ nm ca. 15 min after color development. The detection limits are 50 ng substance per chromatogram zone for gentamycin C_{1a} (*hR_f* 35–40), C₂ + C_{2a} (*hR_f* 40–45), and C₁ (*hR_f* 45–50) (Fig. 2).

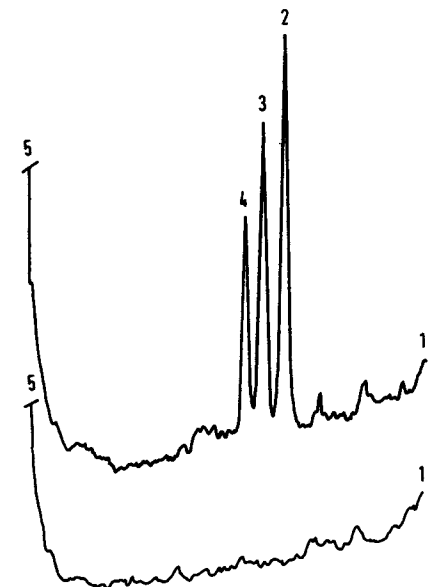


Fig. 2: Absorption scans (A) of a blank and (B) of a gentamycin standard (800 ng substance per application). Start (1), gentamycin C_{1a} (2), gentamycin C₂ + C_{2a} (3), gentamycin C₁ (4), solvent front (5).

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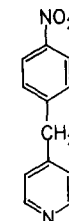
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4-(4-Nitrobenzyl)pyridine Reagent (NBP Reagent)

Reagent for:

- Epoxides
e.g. trichothecene-mycotoxins [1–6]
valepotriates [7, 17]
- Olefins, acetylene derivatives [8]
- 4-Hydroxycoumarin, anthraquinone [8]
- Alkylating agents [9–12]
e.g. sulfur and nitrogen mustard derivatives,
bis-(halogenalkyl)sulfides,
N,N,N-(trihalogenalkyl)amines and
N,N-bis-(halogenalkyl)alkylamines [9, 10],
diazoalkanes and aziridines [10],
substances with labile halogens [12]
- Pyrethroid insecticides
e.g. S-bioallethrine [13]
- Organophosphorus insecticides [16]



$C_{12}H_{10}N_2O_2$
 $M_r = 214.23$

Preparation of Reagent

- Dipping solution I** Dissolve 9 g 4-(4-nitrobenzyl)pyridine (NBP) and 90 mg butylhydroxytoluene (BHT) in 90 ml chloroform.
- Dipping solution II** Dissolve 24 ml tetraethylene pentamine and 60 mg butylhydroxytoluene in 36 ml dichloromethane.
- Spray solution I** Dissolve 2–5 g 4-(4-nitrobenzyl)pyridine in 100 ml acetone [7–10].

Spray solution II Dissolve 92 mg potassium hydrogen phthalate in 100 ml water and adjust the pH to 5.0 with sodium hydroxide solution (1 mol/l). Dissolve 5 g sodium perchlorate in this solution [9].

A sodium acetate buffer solution (0.05 mol/l; pH 4.6) may be employed as an alternative [10].

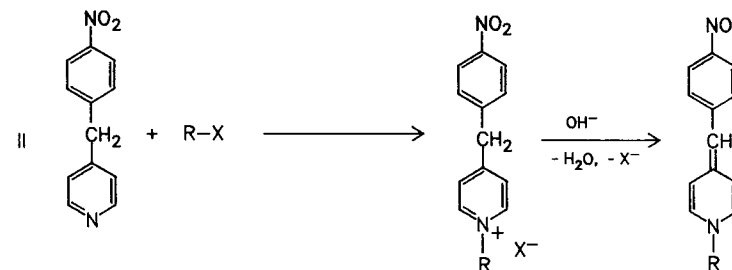
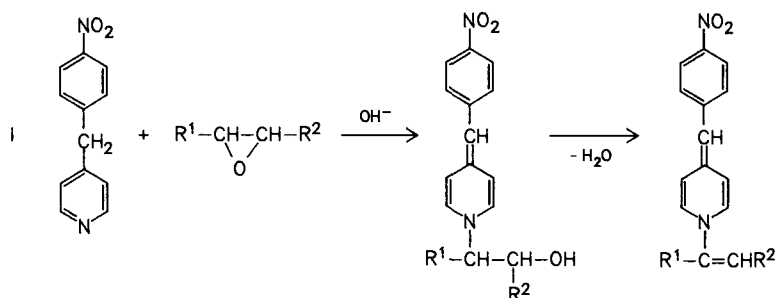
Spray solution III Piperidine [10] or alternatively a mixture of triethylamine and acetone (1 + 4) or sodium hydroxide solution ($c = 10^{-5}$ mol/l; pH 8.5–9) [8].

Storage All solutions may be stored for several days in the refrigerator.

Substances 4-(4-Nitrobenzyl)pyridine
Tetraethylene pentamine
Chloroform
Dichloromethane
Acetone
Potassium hydrogen phthalate
Sodium perchlorate monohydrate
Sodium hydroxide solution (1 mol/l)
Piperidine
Triethylamine
Butylhydroxytoluene
(= 2,6-di-*tert*-butyl-4-methyl phenol)

Reaction

NBP reacts with epoxides according to I to yield methine dyestuffs [14] and with alkylating agents (R-X, X = e.g. halogen) according to II to yield colored pigments [15].



Method

1. *Epoxides*: The chromatograms are dried in a stream of warm air, immersed in dipping solution I for 1–2 s and then heated to 120–150°C for 15–30 min. They are then cooled to room temperature and immersed for 1–2 s in dipping solution II [1].

2. *Acetylene derivatives and substances containing labile halogen*: The dried chromatograms are homogeneously sprayed with spray solution I, dried in a stream of hot air and sprayed once more with the same solution. The color can then be intensified by spraying with sodium hydroxide solution (10^{-5} mol/l) [8].

3. *Alkylating agents*: The chromatograms are freed from mobile phase, then sprayed homogeneously with spray solution I, dried briefly in a stream of cold air, sprayed homogeneously with spray solution II and then heated to 105–140°C for 10–25 min. After cooling to room temperature they are then sprayed with spray solution III [9, 10].

4. *Organophosphorus insecticides*: The chromatograms are freed from mobile phase, immersed in dipping solution I for 10 s and exposed to a saturated acetic anhydride atmosphere for 15 s. After heating to 110°C for 30 min the chromatogram is immersed for 10 s in dipping solution II and dried for a few minutes in a stream of cold air [16].

Trichothecenes, valepotriates, organophosphorus insecticides and alkylating agents yield blue [1, 7, 9, 16] and acetylene derivatives and substances containing labile halogens yellow to red-violet [8, 13] chromatogram zones on a colorless background. The colors produced fade after a short period [7, 10]. By subsequently dipping the dried chromatogram in liquid paraffin – n-hexane (1 + 2; containing 0.1% butylhydroxytoluene) for 2 s and drying the chromatogram once more in a stream of cold air the intensity of the colored chromatogram zones is increased and stabilized for more than 45 min.

Note: Trichothecenes and valepotriates only react when there is an epoxy group present in the molecule; their detection limits are in the range 25–200 ng substance per chromatogram zone [1]. The detection limits for acetylene derivatives are 100–800 ng substance per chromatogram zone, but not all give a positive reaction [8].

Spray solution I can also be employed as a dipping solution. Dipping solution II can be replaced by a freshly made up 10% methanolic solution of anhydrous piperazine.

The reagent can be employed on silica gel, kieselguhr, Si 50 000, cellulose, CN and RP layers. NH_2 layers are unsuitable, since no coloration is produced, as are Nano-SIL C_{18} UV₂₅₄ plates (MACHERY-NAGEL), since the whole plate background is colored violet.

Procedure Tested

Organophosphorus Insecticides [16]

Method	Ascending, one-dimensional stepwise development in a twin trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).

Mobile phase	Chloroform – diethylether – toluene – <i>n</i> -hexane (29.3 + 25.7 + 20 + 25).
Migration distance	a. 6 cm; b. 2 cm
Running time	a. 20 min; b. 5 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air for 3 min and then immersed in dipping solution I for 10 s and exposed to a saturated acetic anhydride atmosphere for 15 s. After heating to 110°C for 30 min the chromatogram was immersed for 10 s in dipping solution II and dried in a stream of cold air. By subsequent dipping the plate into liquid paraffin – *n*-hexane (1 + 2 v/v; containing 0.1% butylhydroxytoluene) for 2 s and drying in a stream of cold air the intensity of the blue to violet chromatogram zones is increased and stabilized for more than 45 min.

By this procedure it is possible to derivatize all of the 10 investigated organophosphorus insecticides (Fig. 1).

In situ quantitation: The direct quantitative determination of the blue-violet derivatives should be made in reflectance at $\lambda = 580 \text{ nm}$.

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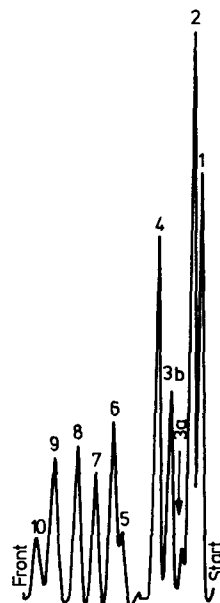
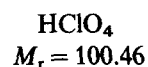


Fig. 1: Absorption scan of a chromatogram track with 150 ng of each substance per chromatogram zone: 1 = demeton-S-methylsulfone, 2 = dimethoate, 3a = transmevinphos, 3b = cis-mevinphos, 4 = demeton-S-methyl, 5 = triazophos, 6 = azinphosmethyl, 7 = azinphos-ethyl, 8 = malathion, 9 = parathion-methyl and 10 = parathionethyl.

Perchloric Acid Reagent

Reagent for:

- Steroids [1–8]
- Bile acids [9, 10]
- Polystyrenes [11]
- Antiepileptics [12–15]
e.g. carbamazepine, primidone
- Psychopharmaceuticals [12, 16]
e.g. chlorodiazepoxide, diazepam,
nitrazepam, oxazepam
- Phenobarbital [12]
- Fatty acid esters [17]
- Nucleosides, nucleotides [18]
- Sugars [19, 20]
- Indoles
e.g. tryptophan, tryptamine [21]



Preparation of Reagent

Dipping solution Add 50 ml perchloric acid (70%) carefully to 50 ml water and after cooling dilute the mixture with 50 ml ethanol.

Spray solution I For steroids: 20% aqueous perchloric acid.

Spray solution II For bile acids: 60% aqueous perchloric acid.

Storage The reagent solutions may be stored for an extended period of time.

Substances Perchloric acid (60%)
Perchloric acid (70%)
Ethanol

Reaction

The reaction mechanism has not been elucidated.

Method

The chromatogram is freed from solvent, dipped in the reagent solution for 5–10 s and then heated to 120–150°C for 5–10 min. (Caution: Remove perchloric acid from the back of the chromatographic plate!).

Mainly colored zones are produced on a colorless background; they are fluorescent under long-wavelength UV light ($\lambda = 365 \text{ nm}$) and are suitable for quantitative analysis [9, 10].

Bile acids also yield fluorescence when an only 5% perchloric acid is employed as reagent and the chromatogram is only heated to 100°C until coloration commences [9]. Steroids can also be detected with 2% methanolic perchloric acid [4].

Note: Heating for too long and to too high a temperature can lead to charring of the substances. For this reason heating, for example, to 80°C for 30 min has also been recommended [11]. The dipping solution can also be employed as a spray solution.

Danger warning: Perchloric acid sprays can condense in the exhausts of fume cupboards and lead to uncontrolled explosions! Dipping is to be preferred for this reason.

The reagent can be employed on silica gel, kieselguhr and Si 50 000 layers.

Procedure Tested

Carbamazepine [13, 14]

Method Ascending, one-dimensional development in a trough chamber. After application of the samples the TLC plate was equilibrated in a chamber at 42% relative humidity for ca. 30 min and then developed immediately.

Layer	HPTLC plates Silica gel 60 (MERCK). Before application of the samples the plates were prewashed three times with chloroform – methanol (1 + 1) and dried at 110°C for 30 min after each wash.
Mobile phase	Chloroform – acetone (16 + 3).
Migration distance	7 cm
Running time	20 min

Detection and result: The chromatogram was dried in a stream of cold air, immersed in the reagent solution for 5 s, dried in the air and then heated to 120°C for 7 min. It could be inspected after allowing to cool for 30 min. Carbamazepine (R_f 30–35) fluoresced blue in long-wavelength UV light ($\lambda = 365$ nm).

The fluorescence was stabilized and enhanced by a factor of 30 by dipping into a solution of liquid paraffin – chloroform – triethanolamine (10 + 60 + 10).

The detection limit for carbamazepine was 50 pg per chromatogram zone.

In situ quantitation: The direct fluorimetric analysis was carried out under long-wavelength UV light ($\lambda_{exc} = 365$ nm; $\lambda_{fl} > 430$ nm, Fig. 1).



Fig. 1: Fluorescence scan of (A) a blank and (B) a carbamazepine standard with 200 ng per chromatogram zone. Start (1), carbamazepine (2), solvent front (3).

References

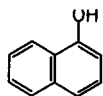
- [1] Metz, H.: *Naturwissenschaften* **1961**, *48*, 569–570.
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Peroxide Reagent

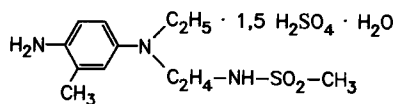
(1-Naphthol — N⁴-Ethyl-N⁴-(2-methanesulfonamidoethyl)-2-methyl-1,4-phenylenediamine Reagent)

Reagent for:

- Peroxides [1]
e.g. hydrogen peroxide, per acids, diacylperoxides, hydroperoxides, ketone peroxides [1]



$C_{10}H_8O$
 $M_r = 144.17$
1-Naphthol



$C_{12}H_{21}N_3O_2S \cdot 1.5 H_2SO_4 \cdot H_2O$
 $M_r = 436.25$
Color developer 3

Preparation of Reagent

Dipping solution Dissolve 3 g 1-naphthol in 150 ml methanol and add 1350 ml water. Dissolve 0.5 g potassium disulfite (potassium metabisulfite) in this solution, add 20 ml glacial acetic acid and dissolve 0.5 g iron(II) sulfate $\cdot 7H_2O$ followed by 2.2 g N⁴-ethyl-N⁴-(2-methanesulfonamidoethyl)-2-methyl-1,4-phenylenediamine (sesquisulfate, monohydrate) (color developer 3, MERCK). Care should be taken at each step that the solution has clarified before adding further components.

Storage

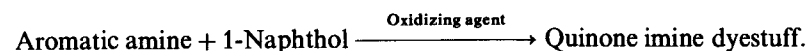
The dipping solution may be used for ca. 1 week. Later it slowly turns blue causing the plate background to deteriorate.

Substances

1-Naphthol
Color developer 3
Potassium disulfite
Iron(II) sulfate heptahydrate
Methanol
Acetic acid (glacial acetic acid)

Reaction

Under the influence of peroxides aromatic amines (color developer 3) react with phenols to yield quinone imines [1].



Method

The chromatograms are freed from mobile phase, immersed in reagent solution for 1 s or sprayed evenly with it and dried in a stream of cold air. Blue chromatogram zones develop in a few minutes on a pale reddish background.

Note: The detection limits for the various peroxides are from 0.5 to 2 μ g substance per chromatogram zone [1]. The reaction works best when the reagent solution is 3–5 days old; later the background absorption increases. The background coloration that is produced on drying in a stream of cold air can be largely avoided by drying the plate after dipping in the absence of oxygen, first with a moist and then with a stream of dry nitrogen [1].

The reagent can be employed on silica gel, kieselguhr, Si 50 000 and particularly sensitively [1] on cellulose layers.

Procedure Tested

Dibenzoyl Peroxide in Acne Preparations [2]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene – dichloromethane – glacial acetic acid (50+2+1).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air, immersed in the reagent solution for 1 s and then dried in a stream of cold air for 15 min. At first reddish and then after 60 min violet chromatogram zones developed on a pink background (detection limit of dibenzoyl peroxide: ca. 500 ng).

Note: The background coloration could be avoided if the dipped chromatogram was stored in a chamber first over streaming moist nitrogen gas for 15 min and

then for a further 15 min over dry nitrogen gas. The zone intensity was then ca. 30% less than when a stream of cold air was employed for drying (Fig. 1).

In situ quantitation: The direct analysis was carried out at $\lambda = 510$ nm if the zones were red in color and at $\lambda = 620$ nm if they were violet.

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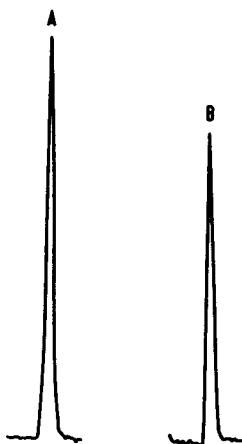
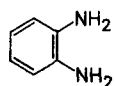


Fig. 1: Reflectance scans of 5 µg dibenzoyl peroxide ($\lambda = 620$ nm); after dipping the chromatogram was dried in (A) a stream of cold air and (B) in a stream of nitrogen.

1,2-Phenylenediamine — Trichloroacetic Acid Reagent

Reagent for:

- α -Keto acids
e.g. ascorbic acid,
dehydroascorbic acid [1]
- Sugars [2]



$C_6H_8N_2$
 $M_r = 108.14$
1,2-Phenylene-
diamine

CCl_3COOH

$C_2HCl_3O_2$
 $M_r = 163.39$
Trichloro-
acetic acid

Preparation of Reagent

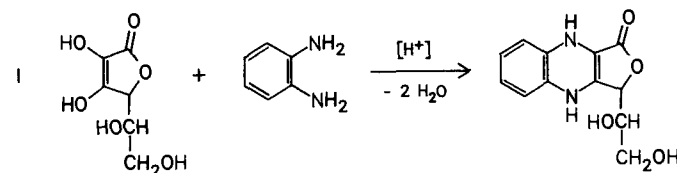
Dipping solution Dissolve 50 mg 1,2-phenylenediamine in 50 ml 10% ethanolic trichloroacetic acid.

Storage The reagent solution should always be made up freshly.

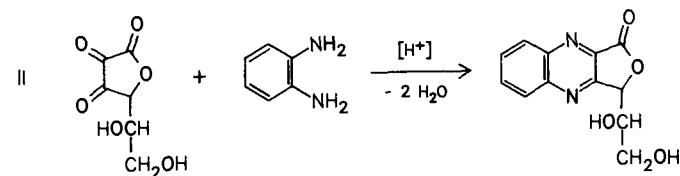
Substances 1,2-Phenylenediamine
Trichloroacetic acid
Ethanol

Reaction

The following reactions are to be expected with ascorbic acid and dehydroascorbic acid:



1. Ascorbic acid



2. Dehydroascorbic acid

Method

The chromatogram is freed from mobile phase in a stream of cold air, either immersed in the reagent solution for 5 s or homogeneously sprayed with it and then heated to 110–120°C for 10–15 min [2].

Chromatogram zones are produced which exhibit a green fluorescence under long-wavelength UV light ($\lambda = 365$ nm).

Note: The chromatogram can be dipped into liquid paraffin — *n*-hexane (1 + 2) for 1 s to stabilize the fluorescence.

The reagent can be employed on silica gel, and cellulose layers.

Procedure Tested

Ascorbic Acid and Dehydroascorbic Acid [1]

Method	Ascending, one-dimensional development in a twin-trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK). Before application of the samples the layer was prewashed once with the mobile phase and dried at 110°C for 20 min. Before it was placed in the developing chamber the prepared HPTLC plate was preconditioned for 30 min at 0% relative humidity (over conc. sulfuric acid).
Mobile phase	Acetone — toluene — formic acid (60 + 30 + 10).
Migration distance	6 cm
Running time	11 min

Detection and result: The chromatogram was dried in a stream of cold air for 2 min, immersed in the dipping solution for 5 s and then heated to 110°C for

10 min. After cooling it was immersed for 1 s in liquid paraffin — *n*-hexane (1 + 2) to stabilize the fluorescence.

In long-wavelength UV light ($\lambda = 365$ nm) ascorbic acid (hR_f 45–50) and dehydroascorbic acid (hR_f 50–55) yielded green fluorescent zones on an orange-colored background. The detection limits were less than 5 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric determination was carried out under long-wavelength UV light ($\lambda_{exc} = 365$ nm, $\lambda_{fl} = 546$ nm, monochromatic filter; Fig. 1).

References

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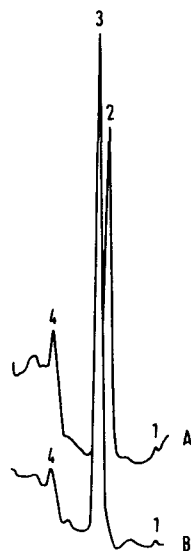


Fig. 1: Fluorescence scans of chromatogram tracks with 200 ng ascorbic acid (A) and 200 ng dehydroascorbic acid (B): Start (1), ascorbic acid (2), dehydroascorbic acid (3), β -front (4).

Phosphomolybdic Acid Reagent

Reagent for:

- Reducing substances
e.g. antioxidants [1–3], ascorbic acid [4],
isoascorbic acid [4, 5], vitamin E [1]
- Steroids [6–9]
- Bile acids, bile acid conjugates [9, 10]
- Lipids [11–14], phospholipids [11, 12]
- Fatty acids [15] or their methyl esters [16]
- Triglycerides [14, 15]
- Subst. phenols [5]
- Indole derivatives [5]
- Prostaglandins [17]
- Components of essential oils
e.g. carvone, agarofuran [18]
- Morphine [5]



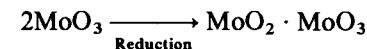
$$M_r = 1825.28$$

Preparation of Reagent

Dipping solution	Dissolve 250 mg phosphomolybdic acid (molybdatophosphoric acid) in 50 ml ethanol.
Spray solution	Dissolve 2–20 g phosphomolybdic acid in 100 ml ethanol [1–3, 6–11, 13, 16], in 2-propanol – methanol (70 + 30) [14] or in water [5, 12].
Storage	The solutions may be stored in the dark for ca. 10 days [14].
Substances	Molybdatophosphoric acid hydrate Ethanol

Reaction

A large number of organic substances can be oxidized with phosphomolybdic acid, whereby a portion of the Mo(VI) is reduced to Mo(IV), which forms blue-grey mixed oxides with the remaining Mo(VI).



Method

Dry the chromatogram in a stream of warm air and immerse for 2–3 s in the reagent solution or spray the layer with it until this acquires an even yellow coloration and dry in a stream of warm air (ca. 2 min).

Blue zones appear immediately or after a few minutes on a yellow background. The background can then be lightened [7] by placing the chromatogram in a twin-trough chamber whose second trough contains 25% ammonia solution.

Note: Occasionally in order to achieve optimal color development a brief heating is to be recommended after dipping or spraying (normally at 105–120°C for 10 min, or in the case of saturated lipids at 150–180°C). This also causes sugars to yield blue derivatives. Heating for too long can result in the background being darkened [11]. The detection sensitivity can be improved by adding 4 ml conc. hydrochloric acid to every 100 ml reagent solution.

The detection limit per chromatogram zone is 50–200 ng for lipids [11], 200–400 ng for antioxidants [3] and several ng for ascorbic acid.

The phosphomolybdic acid reagent can be employed on silica gel, aluminium oxide, polyamide, RP-2, RP-18 and cellulose phases and also on silver nitrate-impregnated silica gel [13].

Procedure Tested

Lecithin, Sphingomyelin [19]

Method	Ascending, one-dimensional development in a linear chamber (CAMAG).
---------------	---------------------------------------------------------------------

Layer	HPTLC plates Silica gel 60 (MERCK). Before applying the sample it was best to prewash the layer with mobile phase (Fig. 1) and then activate at 120°C for 15 min.
Mobile phase	Chloroform – methanol – water (30 + 10 + 1.4).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of cold air for 2 min and sprayed three times with the spray reagent until it began to appear transparent. The plate was dried in cold air after each spray step and finally heated to 120°C for 15 min. Lecithin (R_f 15) and sphingomyelin (R_f 5–10) appeared as dark blue zones on a yellow background (Fig. 1).

In situ quantitation: The photometric analysis was performed in reflectance at $\lambda = 650$ nm.

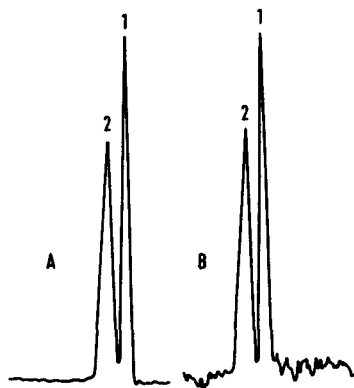


Fig. 1: Reflectance scan of chromatogram tracks (A: layer prewashed with inobile phase, B: with methanol) with 750 ng each substance per chromatogram zone. Sphingomyelin (1), lecithin (2).

References

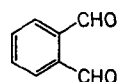
- [1] Seher, A.: *Fette, Seifen, Anstrichm.* **1959**, *61*, 345–351.
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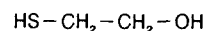
o-Phthalaldehyde Reagent (OPT, OPA)

Reagent for:

- Primary amines [1–6]
e.g. aminoglycoside antibiotics
- Amino acids [1, 2, 7–9]
- Peptides [4, 8, 9]
- Imidazole derivatives [1]
- Indole derivatives
e.g. pindolol [10]
hydroxyindolylacetic acid [11–14]
bufotenine [14, 15]
serotonin, 5-hydroxytryptamine [11, 14–17]
- Ergot alkaloids [18, 19]



$C_8H_6O_2$
 $M_r = 134.14$
o-Phthalal-
dehyde



C_2H_6OS
 $M_r = 78.13$
2-Mercapto-
ethanol

Preparation of Reagent

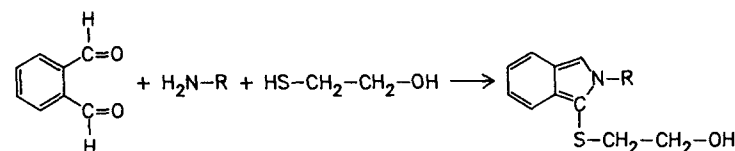
Dipping solution Make 0.1 g o-phthalaldehyde (phthaldialdehyde, OPA) and 0.1 ml 2-mercaptoethanol (2-hydroxy-1-ethanethiol) up to 100 ml with acetone.

Storage The reagent solution is stable for several days when stored in the dark at room temperature.

Substances Phthaldialdehyde
2-Mercaptoethanol
Acetone

Reaction

In the presence of 2-mercaptoethanol o-phthalaldehyde reacts with primary amines to yield fluorescent isoindole derivatives [20]:



o-Phthalaldehyde Amine 2-Mercaptoethanol Fluorescing derivative

Method

Immerse the dried chromatogram for 1 s in the reagent solution and then heat to 40–50 °C in the drying cupboard for 10 min.

Substance zones are produced that mainly yield blue fluorescence under long-wavelength UV light ($\lambda = 365$ nm) (indoles occasionally fluoresce yellow [15]), colored zones are also produced occasionally. The fluorescence is stabilized by immersing in 20% methanolic polyethylene glycol solution [5].

For the detection of the ergot alkaloids 0.2 g o-phthalaldehyde in 100 ml conc. sulfuric acid (!) [15] or buffer solution is employed as spray solution. Cysteine [11, 12, 15, 19] or sulfurous acid [17] is occasionally substituted for mercaptoethanol.

Note: The reagent can be employed on silica gel, alumina and silica gel plates but not on amino or polyamide layers. The dipping solution can also be employed as a spray solution.

If the reaction proves difficult the TLC plate should first be dipped in 1% solution of triethylamine in acetone or in a solution of 1 to 2 drops sodium hydroxide solution ($c = 10$ mol/l) in methanol to optimize the pH for the reaction. This effect can also be achieved by employing borate buffer, pH = 11, instead of acetone in the spray reagent [10, 11].

Since the fluorescence intensity of the zones on silica gel layers is reduced after a few minutes the determination of aromatic amino acids is usually performed on

cellulose layers, where the fluorescence remains stable for a longer period of time [8]. Under these conditions proline can still be detected after heating the plate to 110°C for 1 h, while the other zones fade.

The detection limits for amino acids and peptides are between 50 and 200 pmol per chromatogram zone [9], 400 pg for 5-hydroxyindolylacetic acid [11] and 300 pg for dihydroxyergotoxin [19].

Procedure Tested

Gentamycin C Complex [21]

Method	Ascending, one-dimensional development in a twin-trough chamber (CAMAG) with 5 ml ammonia solution (25%) in the trough free from mobile phase. Chamber saturation: ca. 15 min; development at 10–12°C.
Layer	HPTLC plates Silica gel 60 (MERCK), prewashed by developing three times with chloroform – methanol (1 + 1) and drying at 110°C for 30 min after each development.
Mobile phase	Chloroform – ethanol – ammonia solution (25%) (10 + 9 + 10); the lower organic phase was used for chromatography.
Migration distance	ca. 5 cm
Running time	ca. 20 min

Detection and result: The chromatogram was freed from mobile phase for ca. 45 min in a current of cold air, immersed for 1 s in the reagent solution and dried in the air.

Alongside gentamycin C₁ (*hR_f* 45–50) and gentamycin C_{1a} (*hR_f* 35–40), gentamycins C₂ and C_{2a} formed a common zone (*hR_f* 40–45; Fig. 1).

Quantitation could be performed fluorimetrically: $\lambda_{\text{exc}} = 313 \text{ nm}$, $\lambda_{\text{fl}} > 390 \text{ nm}$. The chromatogram was first immersed for 1 s in a solution of liquid paraffin – *n*-hexane (1 + 2) to stabilize the fluorescence. The detection limit was ca. 30 ng per chromatogram zone for each substance.

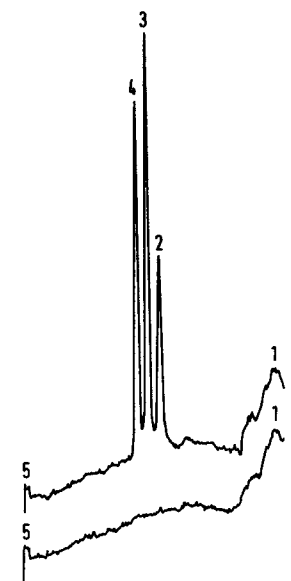


Fig. 1: Fluorescence scan of (A) a blank track and (B) a standard gentamycin mixture (800 ng C complex per application). Start (1), gentamycin C_{1a} (2), C₂/C_{2a} (3), C₁ (4), solvent front (5).

References

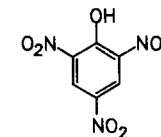
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Picric Acid – Perchloric Acid Reagent

Reagent for:

- Δ^5 -3 β -Hydroxysteroids [1]
- Sterols
e.g. cholesterol [1, 2]
coprostanol [2]



$C_6H_3N_3O_7$
 $M_r = 229.11$
 Picric acid

$HClO_4$
 $M_r = 100.47$
 Perchloric acid

Preparation of Reagent

Dipping solution Dissolve 100 mg picric acid (2,4,6-trinitrophenol) in 36 ml acetic acid (96%) and carefully add 6 ml perchloric acid (70%).

Storage The dipping solution should be freshly made up before use.

Substances Picric acid
 Acetic acid (96%)
 Perchloric acid (70%)

Reaction

Presumably perchloric acid oxidizes the steroids at ring A and these then form charge transfer complexes with picric acid.

Method

After drying in a stream of cold air the chromatogram is immersed in the reagent solution for 1 s and heated to 70–75°C for 3–5 min (until the color develops optimal intensity). Red-colored zones are usually formed on a white background; occasionally, however, yellow to brown chromatogram zones, which gradually fade, are formed on a white to pale yellow background [1].

Note: The reagent can be employed on silica gel and cellulose layers. The coloration of the stained chromatogram zones is dependent on the temperature and duration of heating. For instance, cholesterol appears bluish-pink after heating to 75–80°C for 3–5 min [1], but yellow to brown-colored after heating for 20–30 min (cf. "Procedure Tested").

Warning of danger: The perchloric acid-containing reagent should not be employed as a spray solution for reasons of safety.

Procedure Tested

Cholesterol, Coprostanol [2]

Method	Ascending, one-dimensional development in a trough chamber.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK). The plates were prewashed by developing once to the upper edge with chloroform — methanol (1 + 1) and then activated at 110°C for 30 min.
Mobile phase	Cyclohexane — diethyl ether (10 + 10).
Migration distance	6 cm
Running time	ca. 15 min

Detection and result: The developed chromatogram was dried in a stream of cold air, immersed in the reagent solution for 1 s and heated to 80°C for 20–30 min (until optimal color development occurred). Yellow to brown-colored zones were produced on a pale yellow-colored background; these were suitable for quantitative analysis. The detection limits for cholesterol (hR_f 20–25) and coprostanol 25–30) were a few nanograms per chromatogram zone.

In situ quantitation: The absorption-photometric analysis was carried out at $\lambda = 378$ nm.

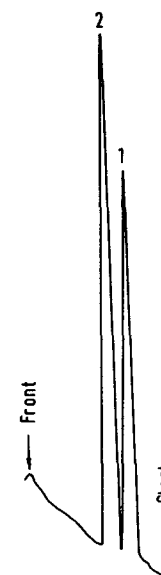


Fig. 1: Absorption scan of a chromatogram track with 250 ng cholesterol and 500 ng coprostanol per chromatogram zone. Cholesterol (1), coprostanol (2).

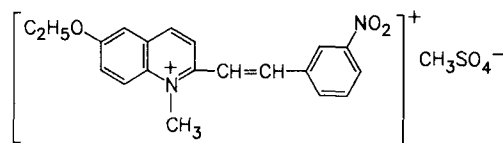
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Pinacryptol Yellow Reagent

Reagent for:

- Anionic active and nonionogenic detergents [1 – 7]
- Organic anions of aliphatic phosphates, phosphonates, sulfates, sulfonates and sulfamates [8, 9]
- Sweeteners
e.g. cyclamate, saccharin, dulcin [10]



$C_{21}H_{22}N_2O_7S$
 $M_r = 446.48$

Method

The chromatogram is freed from mobile phase in a stream of warm air and either immersed for 2 s in the dipping solution or homogeneously sprayed with it until the layer begins to be transparent. In the case of detergents the chromatograms are evaluated while still moist [3], in the case of sweeteners after drying for 10 min in the dark [10].

Fluorescent chromatogram zones are produced on a dark or fluorescent background under long-wavelength ($\lambda = 365$ nm) and occasionally short-wavelength UV light ($\lambda = 254$ nm).

Note: Detergents fluoresce blue, yellow or orange [1, 3, 5, 6]. On cellulose layers aliphatic organic anions with chain lengths of at least 3 C atoms yield yellow to orange fluorescence on a pale green background; increasing chainlength of the organic part of the molecule has a positive effect and hydroxyl groups a negative effect on the fluorescence intensity [8]. Inorganic anions, carboxylic acids and amino acids do not react [8]. Cyclamate fluoresces orange and saccharin and dulcin can be recognized as orange and dark violet zones respectively [10].

The reported detection limits per chromatogram zone are 5 – 50 μ g for detergents [3, 5], 0.025 – 5 μ g for aliphatic phosphate, phosphonate, sulfate, sulfonate and sulfamate ions [8] and 0.2 – 1 μ g for sweeteners [10].

The reagent can be employed on silica gel, cellulose, polyamide and alumina layers.

Procedure Tested

Sweeteners [10, 11]

Method	Ascending, one-dimensional double development in a trough chamber with chamber saturation.
Layer	HPTLC plates Cellulose (MERCK).
Mobile phase	Ethyl acetate – acetone – ammonia (25%) (6 + 6 + 1).
Migration distance	2 × 8 cm
Running time	2 × 10 min with 5 min intermediate drying in a stream of warm air.

Preparation of Reagent

Dipping solution	Dissolve 100 mg pinacryptol yellow in 100 ml ethanol (95%) with gentle heating.
Spray solution	Dissolve 100 mg pinacryptol yellow in 100 ml water with boiling [2, 3] or in ethanol (95%) with heating [8, 10].
Storage	The reagent solution may be stored for several months in the dark.
Substances	Pinacryptol yellow Ethanol

Reaction

The reaction has not been elucidated. An exchange of counterions is probable in the case of anionic compounds [8].

Detection and result: The HPTLC plate was freed from mobile phase, immersed in the reagent solution for 2 s, dried for a few seconds in a stream of cold air and then immediately stored in the dark for 10 min.

In long-wavelength UV light ($\lambda = 365$ nm) cyclamate (hR_f 5–10) appeared as a light orange fluorescent zone while saccharin (hR_f 15–20) and acesulfame (hR_f 25–30) yielded violet and brownish zones respectively on a pale blue fluorescent background. Under the chromatographic conditions chosen dulcin lay in the region of the front. The fluorescence colors are concentration-dependent. At $\lambda = 254$ nm the sweeteners were detectable as weak light fluorescent zones.

In situ quantitation: The fluorimetric determination was carried out at $\lambda_{exc} = 313$ nm and $\lambda_{fl} = 365$ nm (monochromatic filter M 365; Fig. 1).

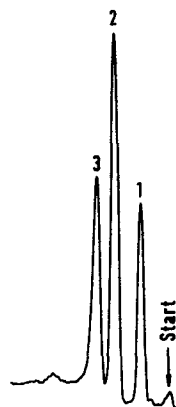


Fig. 1: Fluorescence scan of a chromatogram track with 3 μ g each of acesulfame and saccharin and 2.25 μ g of cyclamate per chromatogram zone. Cyclamate (1), saccharin (2), acesulfame (3).

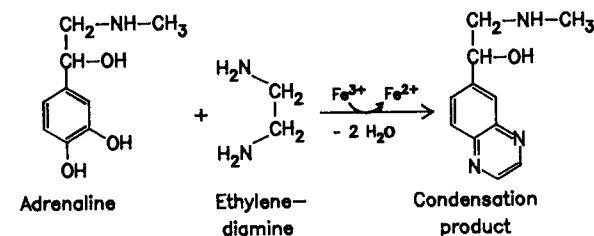
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Potassium Hexacyanoferrate(III) – Ethylenediamine Reagent

Reagent for:

- | | | |
|---------------------------------------|--------------------------------------|---------------------------------------------------------|
| • Catecholamines [1–4] | | $\text{H}_2\text{N}-\text{CH}_2\text{CH}_2-\text{NH}_2$ |
| • 3,4-Dihydroxyphenyl-acetic acid [5] | $\text{K}_3(\text{Fe}(\text{CN})_6)$ | $\text{C}_2\text{H}_8\text{N}_2$ |
| | $M_r = 329.26$ | $M_r = 60.10$ |
| • 3-Hydroxytyramine [5] | Potassium hexacyano-ferrate(III) | Ethylenediamine |



Method

The chromatograms are dried thoroughly in a stream of warm air, immersed in the reagent solution for 1 s, dried in the air and heated in the drying cupboard at 65 °C for 30 min.

Fluorescent zones, which are suitable for quantitation [2], are visible in long-wavelength UV light ($\lambda \geq 400$ nm).

Note: The dipping solution may also be used as a spray solution [2]. Catecholamines are only separable on silica gel layers as their triacetyl derivatives but they can be separated underivatized on cellulose layers [4].

Procedure Tested

Adrenaline, Noradrenaline, Dopamine, Dopa as Triacetyl Derivatives [6]

Method

Ascending, one-dimensional development in a trough chamber. After application of the sample the layer was equilibrated for 30 min in a conditioning chamber at 18% relative humidity and then developed immediately.

Layer

HPTLC plates Silica gel 60 (MERCK).

Mobile phase

Acetone – dichloromethane – formic acid (50 + 50 + 1).

Migration distance

8 cm

Running time

20 min

Detection and result: The chromatogram was dried in a stream of warm air, immersed in the freshly prepared reagent solution for 1 s and then heated to 80 °C for ca. 15 min. Blue-yellow fluorescent zones were visible under long-wavelength UV light ($\lambda = 365$ nm).

Preparation of Reagent

Dipping solution Mix 20 ml methanol and 5 ml ethylenediamine and then add 5 ml of a solution of 0.5 g potassium hexacyanoferrate(III) in 100 ml water.

Storage The solution may be kept in the refrigerator for 1 week; however, it should always be freshly prepared for in-situ quantitation [3].

Substances Methanol
Ethylenediamine
Potassium hexacyanoferrate(III)

Reaction

On oxidation by potassium hexacyanoferrate(III) adrenaline is converted into adrenochrome which then condenses with ethylenediamine:

In situ quantitation: The fluorimetric analysis was performed at $\lambda_{exc} = 405$ nm and the fluorescent light was measured at $\lambda_{em} > 460$ nm. The detection limits were 200 pg dopamine (hR_f 65–70), 400 pg adrenaline (hR_f 55–60) or noradrenaline (hR_f 45–50) and 1–2 ng dopa (hR_f 80–85) per chromatogram zone (Fig. 1).

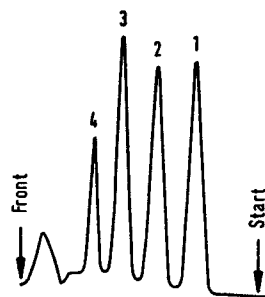


Fig. 1: Fluorescence scan of the catecholamine derivatives (each ca. 10 ng) of noradrenaline (1), adrenaline (2), dopamine (3), dopa (4).

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Potassium Hexacyanoferrate(III) – Sodium Hydroxide Reagent

Reagent for:

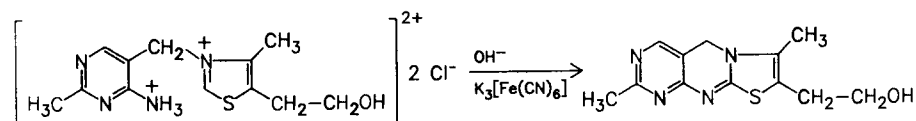
- Vitamin B₁ and its phosphates [1–2] $K_3(Fe(CN)_6)$
- Adrenaline, noradrenaline, isoprenaline [3] $M_r = 329.25$

Preparation of Reagent

- Dipping solution** First dissolve 10 mg potassium hexacyanoferrate(III) and then 1 g sodium hydroxide pellets in 7 ml water and then dilute the solution with 20 ml ethanol.
- Storage** The dipping solution should be prepared fresh before use, since it is unstable.
- Substances** Potassium hexacyanoferrate(III)
Sodium hydroxide pellets
Ethanol

Reaction

Potassium hexacyanoferrate(III) forms, for example, fluorescent thiochrome with vitamin B₁:



Method

The developed chromatograms are dried in a stream of warm air for 1 min, then after cooling they are immersed in the reagent solution for 1 s and dried again in a stream of warm air for 30 s.

The dipping solution can also be used as a spray solution; other concentrations are also reported in the literature [1–3].

Vitamin B₁ and its phosphates yield bluish to bright blue fluorescent zones in long-wavelength UV light ($\lambda = 365$ nm). But adrenaline, noradrenaline, dopa and isoprenaline yield red colors and dopamine blue. Noradrenaline yields an orange-colored fluorescence in long-wavelength UV light ($\lambda = 365$ nm).

Note: The reagent may be applied to silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Vitamin B₁ [2]

Method	Ascending, one-dimensional development in a twin-trough chamber with chamber saturation. After application of the samples the layer was equilibrated for 15 min in the mobile phase-free part of the twin-trough chamber.
Layer	HPTLC plates Silica gel 60 (MERCK) which were prewashed once to the upper edge of the plate with chloroform – methanol (1 + 1) and then dried at 110 °C for 30 min.
Mobile phase	Methanol – ammonia solution (25%) – glacial acetic acid (8 + 1 + 1).
Migration distance	6 cm
Running time	ca. 20 min

Detection and result: The chromatogram was dried in a stream of warm air for 1 min, after cooling it was immersed for 1 s in the reagent solution. After redrying in a stream of warm air it was dipped into a mixture of chloroform – liquid

paraffin – triethanolamine (6 + 1 + 1) for 1 s to enhance (by a factor of 2) and stabilize the fluorescence and dried again in a stream of warm air for 30 s.

Under long-wavelength UV light ($\lambda = 365$ nm) thiamine (R_f 40–45) appeared as a bluish fluorescent zone which could be employed for quantitative analysis (Fig. 1). The detection limit was 500 pg vitamin B₁ per chromatogram zone.

In situ quantitation: The fluorimetric quantitation took place under long-wavelength UV light ($\lambda_{exc} = 365$ nm, $\lambda_{fl} > 430$ nm).

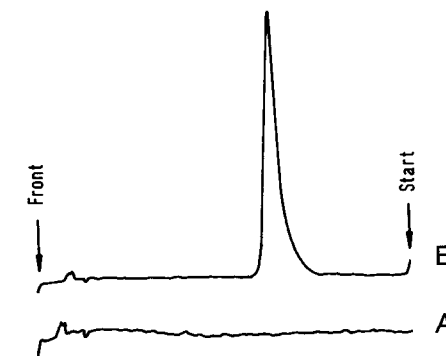


Fig. 1 Fluorescence scan of a blank track (A) and of a chromatogram track with 5 ng thiamine per chromatogram zone (B).

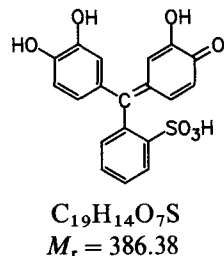
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Pyrocatechol Violet Reagent

Reagent for:

- Metal ions (cations) [1–4]
e.g. tin in organometallic compounds (stabilizers) [1–3]
molybdenum, tungsten [4]



Preparation of Reagent

Dipping solution	Dissolve 100 mg pyrocatechol violet (pyrocatecholsulfophthalein) in 100 ml ethanol.
Storage	The reagent should always be freshly made up.
Substances	Pyrocatechol violet Ethanol (96%)

Reaction

Pyrocatechol violet forms colored complexes with a variety of metal ions, the complexes are stable in differing pH ranges.

Method

The chromatogram is freed from mobile phase, dipped for 1 s in the reagent solution or sprayed evenly with it until the plate begins to be transparent and dried in a stream of cold air.

Colored zones are formed (tin: violet-red to blue) on a yellow ochre background [3].

Note: The dipping solution can also be employed as a spray reagent.

Organotin compounds must first be decomposed before using the reagent [1–3]. This can be done by first heating the chromatogram to 110°C for 30 min and then exposing it to saturated bromine vapors for 60 min (7–9 drops bromine in a chamber), the excess bromine is then allowed to evaporate in the air (10 min) and the plate is treated with the dyeing reagent [1, 3]. Quantitative determinations should be made at $\lambda = 580$ nm, the detection limits for organotin compounds are in the 10–20 ng per chromatogram zone range [3].

The reagent can be employed on silica gel, kieselguhr, Si 50 000 and cellulose layers.

Procedure Tested

Organotin Compounds [3, 5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Methyl isobutyl ketone – pyridine – glacial acetic acid (97.5 + 1.5 + 1).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was freed from mobile phase (heated to 110°C for 30 min) and then exposed to bromine vapor for 1 h in a chamber, after blowing off excess bromine from the layer it was immersed for 1 s in the reagent solution. On drying in air dibutyltin dilaurate (hR_f 25–30), dibutyltin dichloride (hR_f 25–30), dioctyltin oxide (hR_f 40), tributyltin oxide (hR_f 80), tributyltin chloride (hR_f 80) and tetrabutyltin (hR_f 85–90) produced persistent blue zones on a yellow ochre background (Fig. 1).

In situ quantitation: The analysis is performed at $\lambda = 580$ nm. The detection limits for the di- and trialkyltin compounds are 10–20 ng and for tetrabutyltin 500 ng per chromatogram zone (Fig. 2).

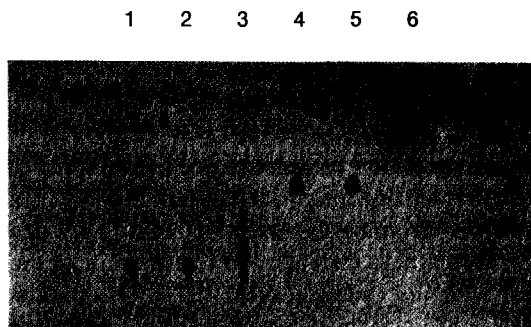


Fig. 1: Chromatogram of organotin compounds. Dibutyltin dilaurate (1), dibutyltin dichloride (2), dioctyltin oxide (3), tributyltin oxide (4), tributyltin chloride (5), tetrabutyltin (6).

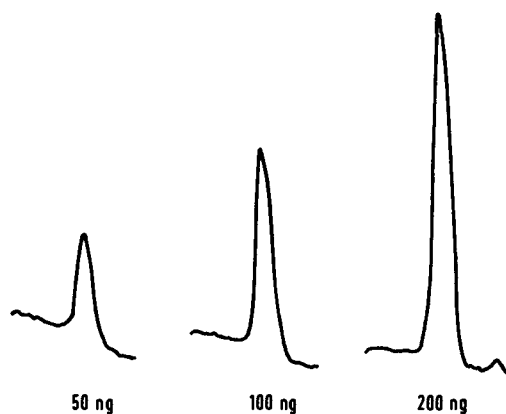


Fig. 2: Absorption scans of chromatograms with 50, 100 and 200 ng dibutyltin dichloride per chromatogram zone.

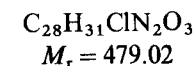
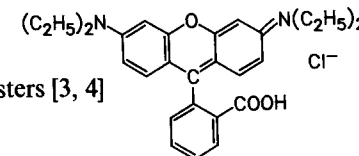
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Rhodamine B Reagent

Reagent for:

- Lipophilic substances, lipids [1–3]
e.g. triglycerides [3], gangliosides [2]
fatty acids and fatty acid methyl esters [3, 4]
fatty alcohol dinitrobenzoates [5]
- Prenols, prenylquinones, prenyl vitamins [6]
- Polyphenols [7]
- Flavonols [8]
- Detergents [9]



Preparation of Reagent

Dipping solution Dissolve 50 mg rhodamine B in 200 ml water.

Spray solution Dissolve 10 to 500 mg rhodamine B in 100 ml ethanol [3, 7], methanol [5] or water [2].

Storage Both reagent solutions may be stored over a long period.

Substances Rhodamine B (C.I. 45170)
Ethanol

Reaction

Because it contains amino and carboxylic groups rhodamine B tends to form zwitter ions which easily associate and can accumulate in lipophilic chromatogram zones.

Method

The chromatograms are freed from mobile phase and immersed in the reagent solution for 1 s or evenly sprayed with it. Red-violet zones are usually formed on a pink background; they fluoresce a deeper red than their surroundings in long-wavelength UV light ($\lambda = 365$ nm).

Note: Rhodamine B is a universal reagent that can be used on silica gel, talc, starch [5] and cellulose layers, just as on urea [1] or silver nitrate-impregnated [7] phases. Liquid paraffin-impregnated silica gel and RP layers are less suitable, since the background to the chromatographic zones is also intensely colored. It is often possible to increase the detection sensitivity by placing the plate in an atmosphere of ammonia after it has been sprayed or dipped, alternatively it can be oversprayed with sodium or potassium hydroxide solution.

Procedure Tested

Fatty Acids [10]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Kieselguhr (MERCK), which were impregnated before application of the samples by a single development in a 1% solution of viscous paraffin oil in petroleum ether.
Mobile phase	Acetic acid – acetone – water (40 + 20 + 4).
Migration distance	17 cm
Running time	60 min

Detection and result: The chromatogram was freed from mobile phase and immersed in the reagent solution for 1 s. Arachidic acid (hR_f 15–20), stearic acid (hR_f 30–35), palmitic acid (hR_f 50–55), myristic acid (hR_f 60–65) and lauric acid (hR_f 70–75) appeared as pink zones on a reddish background.

The reaction was not particularly sensitive on paraffin-impregnated kieselguhr layers because of background coloration. For quantitation it was better to use the five-fold more sensitive rhodamine 6G reagent (q.v.).

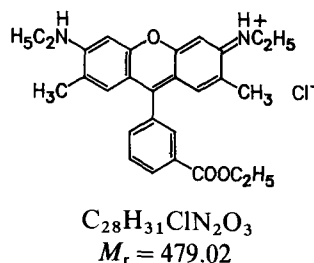
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Rhodamine 6G Reagent

Reagent for:

- Lipophilic substances, lipids [1–11]
e.g. hydrocarbons [1]
fatty acids and fatty acid esters [2, 3]
ubiquinones [4]
gangliosides [5]
steroids, sterols [3, 6]
triterpene alcohols [7]
diglycerides, triglycerides [3, 8]
phospholipids [3]



Method

The chromatograms are freed from mobile phase and immersed in the reagent solution for 1 s or evenly sprayed with it. Pink-colored chromatogram zones are usually formed on a red-violet background, these fluoresce deeper yellow-orange than their environment in long-wavelength UV light ($\lambda = 365$ nm).

Note: Rhodamine 6G is a universal reagent which can also be incorporated in the TLC layers [4, 9] or added to the mobile phase [4]. The spray reagent can also be made up in water [8], acetone [4, 6] or ammonia solution ($c = 2.5$ mol/l) [5]. The visual detection limit is most favorable when the water from the mobile phase or the detection reagent has not completely evaporated from the layer. This can be recognized by the fact that the background fluorescence has not turned from red to pink [4].

It is often possible to increase the detection sensitivity in visible light by exposing the dipped or sprayed chromatogram to ammonia vapors; it can also be sprayed with caustic soda or potash solution. When this is done the fluorescence intensity is reduced on silica gel layers and increased on RP ones.

The reagent can be employed on silica gel, kieselguhr, cellulose and Florisil layers; these can also be impregnated, if desired, with silver nitrate.

Preparation of Reagent

Dipping solution	Dissolve 50 mg rhodamine 6G in 100 ml ethanol (96%).
Spray solution	Dissolve 100 mg rhodamine 6G in 100 ml ethanol (96%).
Storage	Both reagent solutions may be stored for an extended period.
Substances	Rhodamine 6G (C.I. 45160) Ethanol

Reaction

Rhodamine 6G accumulates in lipophilic chromatogram zones giving rise to a stronger fluorescence than in their surroundings.

Procedure Tested

Fatty Acids [10]

Method	Ascending, one-dimensional development in a trough chamber.
Layer	HPTLC plates RP-18 without fluorescence indicator (MERCK).
Mobile phase	Acetone – acetonitril – 0.1 mol/l aqueous lithium chloride solution (10 + 10 + 1).
Migration distance	7 cm
Running time	8 min

Detection and result: The chromatogram was freed from mobile phase and immersed for 1 s in the reagent solution. Arachidic acid (hR_f 35–40), stearic acid

(hR_f 40–45), palmitic acid (hR_f 45–50), myristic acid (hR_f 55–60) and lauric acid (hR_f 60–65) appeared as pink-colored zones on a red-violet background; these fluoresced a deeper yellow-orange than their environment in long-wavelength UV light ($\lambda = 365$ nm) (Fig. 1).

In situ quantitation: The fluorimetric analysis was carried out in long-wavelength UV light ($\lambda_{exc} = 365$ nm; $\lambda_{fl} > 560$ nm). The detection limit for fatty acids was ca. 100 ng per chromatogram zone.

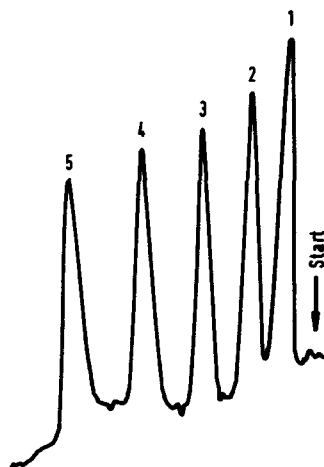


Fig. 1: Fluorescence scan of a fatty acid mixture with 500 ng substance per chromatogram zone. Arachidic acid (1), stearic acid (2), palmitic acid (3), myristic acid (4), lauric acid (5).

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Silver Nitrate — Sodium Hydroxide Reagent

Reagent for:

- Carbohydrates
e.g. mono- and oligosaccharides [1–8]
sugar alcohols [4, 9]

AgNO_3	NaOH
$M_r = 169.87$	$M_r = 40.0$
Silver nitrate	Sodium hydroxide

Preparation of Reagent

Dipping solution I	Make 1 ml of a saturated aqueous silver nitrate solution up to 200 ml with acetone. Redissolve the resulting precipitate by adding 5 ml water and shaking.
Dipping solution II	Dissolve 2 g sodium hydroxide pellets in 2 ml water with heating and make up to 100 ml with methanol.
Storage	Dipping solution I should be prepared freshly each day, dipping solution II may be stored for several days.
Substances	Silver nitrate Sodium hydroxide pellets Acetone Methanol

Reaction

The ionic silver in the reagent is reduced to metallic silver by reducing carbohydrates.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in solution I for 1 s or sprayed evenly with it, then dried in a stream of cold air, immersed in solution II for 1 s or sprayed with it and finally heated to 100 °C for 1–2 min.

Occasionally even without warming brownish-black chromatogram zones are produced on a pale brown background.

Note: The background can be decolorized by spraying afterwards with 5% aqueous ammonia solution and/or 5–10% sodium thiosulfate in 50% aqueous ethanol [2, 3]. The sodium hydroxide may be replaced by potassium hydroxide in dipping solution II [4].

The reagent may be employed on silica gel, cellulose and cellulose acetate layers.

Procedure Tested

Sugar Alcohols [9]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation (15 min).
Layer	HPTLC plates Si 50 000 (MERCK). Before application of the sample the plates were prewashed once by developing in chloroform — methanol (50 + 50) and then dried at 110 °C for 30 min.
Mobile phase	1-Propanol — water (18 + 2).
Migration distance	7 cm
Running time	1 h

Detection and result: The chromatogram was dried in a stream of cold air, immersed in dipping solution I for 1 s, then dried in a stream of cold air, immersed in dipping solution II for 1 s and finally heated to 100 °C for 1–2 min.

The sugar alcohols sorbitol (hR_f 15–20), mannitol (hR_f 20–25) and xylitol (hR_f 30–35) appeared as light to dark brown chromatogram zones on a beige-colored background. The detection limits were 1 ng substance per chromatogram zone.

In situ quantitation: The photometric determination of absorption in reflectance was carried out at a mean wavelength of $\lambda = 530$ nm (Fig. 1).

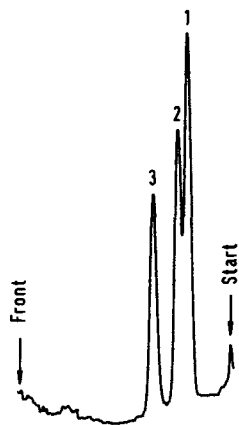


Fig. 1: Reflectance scan of a chromatogram track with 50 ng substance per chromatogram zone: sorbitol (1), mannitol (2), xylitol (3).

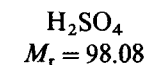
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Sulfuric Acid Reagent

Reagent for:

- Steroids [1 – 3]
 - e.g. estrogens, androgens, anabolics [1 – 4]
 - bile acids [2, 5, 6]
 - cholesterol, cholesteryl esters [7]
- Steroid conjugates [6, 8, 9]
- Sapogenins, saponins [2, 10]
- Spironolactone, canrenone [11]
- Cardiac glycosides [2]
- Alkaloids [2]
- Gibberellins [12]
- Prostaglandins [13]
- Lipids
 - e.g. fatty acids [13], ceramides [14]
 - phospholipids [15]
- Phenothiazines [16]
- Triphenodioxazines [17]
- Mycotoxins
 - e.g. aflatoxins, trichothecenes [12 – 20]
- Antibiotics [21, 22]
- Vitamin A acid [23]



Preparation of Reagent

Dipping solution	Add 10 ml sulfuric acid (95–97%) cautiously to 85 ml water while cooling with ice and add 5 ml methanol after mixing thoroughly.
Spray solution	Add 5–10 ml sulfuric acid cautiously under cooling to 85 to 95 ml acetic anhydride [1], ethanol [1], butanol [5] or methanol [11].
Storage	The dipping solution may be kept at 4°C for an extended period.
Substances	Sulfuric acid (95–97%) Acetic anhydride Methanol Ethanol absolute

Reaction

The reaction mechanism has not yet been elucidated.

Method

The chromatogram is dried in a stream of warm air for 10 min, immersed in the dipping solution for 1–2 s or evenly sprayed with the spray solution, dried in a stream of warm air and then heated to 95–140°C for 1–20 min.

Under long-wavelength UV-light ($\lambda = 365$ nm) characteristic substance-specific yellow, green, red or blue fluorescent chromatogram zones usually appear which are often recognizable in visible light [7] – sometimes even before heating [2] – as colored zones on a colorless background and which are suitable for fluorimetric analysis [1].

Note: Sulfuric acid is a universal reagent, with which almost all classes of substance can be detected by charring at elevated temperatures (150–180°C). The production of colored or fluorescent chromatogram zones at lower temperatures (< 120°C) and their intensities are very dependent on the duration of heating, the

temperature [2] and on the solvent employed [1]. For instance, the detection of prostaglandins is most sensitive after heating to 80°C for only 3–5 min [13].

The following detection limits (substance per chromatogram zone) have been reported: steroids (1–10 ng) [1, 2], steroid conjugates (< 50 ng) [8], prostaglandins (< 1 ng) [13], phenothiazines (2 µg) [16].

The reagent can be used on silica gel, kieselguhr, Si 50000 and RP layers.

Procedure Tested

Cis-/trans-Diethylstilbestrol, Ethinylestradiol [24]

Method	Ascending, one-dimensional development in a twin-trough chamber with the spare trough containing 2 ml (25%) ammonia solution. Before development the HPTLC plate was preconditioned with eluent vapors for ca. 60 min.
Layer	HPTLC plates Silica gel 60 (MERCK); prewashed by triple development with chloroform – methanol (50 + 50) and then heated to 110°C for 30 min.
Mobile phase	Chloroform – methanol (19 + 1).
Migration distance	5 cm
Running time	ca. 10 min

Detection and result: The chromatogram was freed from mobile phase and ammonia vapors and immersed twice for 10 s in reagent solution, with intermediate drying in a stream of cold air, and then heated to 95°C for 12 min. The steroid derivatives were then visible under long-wavelength UV light ($\lambda = 365$ nm) as light blue fluorescent zones on a dark background: cis-diethylstilbestrol (hR_f 15–20), trans-diethylstilbestrol (hR_f 40–45) and ethinylestradiol (hR_f 50–55).

In situ quantitation: The chromatogram was immersed twice for ca. 1 s with brief intermediate drying in a mixture of chloroform – liquid paraffin and triethanolamine (60 + 10 + 10) to stabilize the fluorescence (for ca. 24 h) and increase its intensity (by a factor of ca. 3). The analysis was made in UV light

($\lambda_{exc} = 313$ nm, $\lambda_{fl} > 390$ nm); the detection limit was 500 pg per chromatogram zone (Fig. 1).

Note: The result of the analysis is extremely dependent on the acid concentration employed, the immersion time and the subsequent temperature and duration of heating.

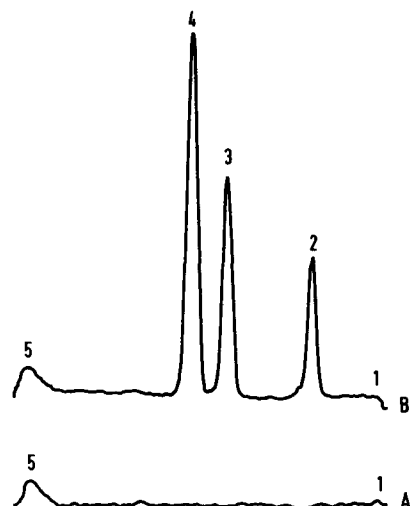


Fig. 1: Fluorescence scan of a blank (A) and of a diethylstilbestrol-ethinylestradiol mixture (B), each with 10 ng substance per chromatogram zone. Start (1), cis-diethylstilbestrol (2), trans-diethylstilbestrol (3) ethinylestradiol (4), front (5).

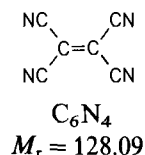
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Tetracyanoethylene Reagent (TCNE Reagent)

Reagent for:

- Aromatic hydrocarbons and heterocyclics [1, 2]
- Aromatic amines and phenols [2, 3]
- Indole derivatives [3, 4, 6]
- Carbazoles [3]
- Phenothiazines [5]

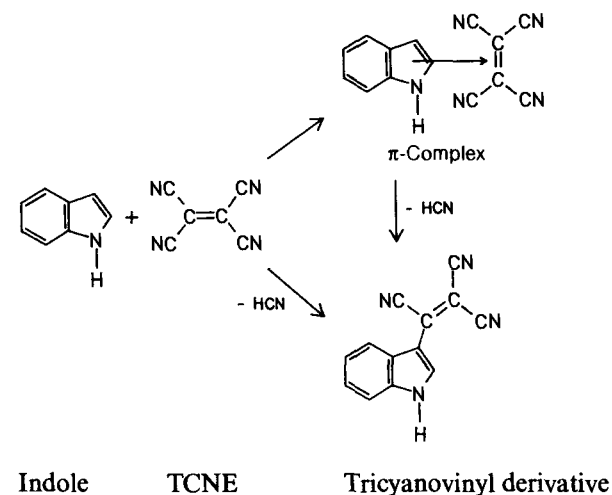


Preparation of Reagent

Dipping solution	Dissolve 0.5 g tetracyanoethylene (TCNE) in 100 ml dichloromethane.
Spray solution	Dissolve 0.5–1 g TCNE in ethyl acetate [3], dichloromethane [4] or acetonitrile [5].
Storage	The reagent solutions may be kept for several days.
Substances	Tetracyanoethylene Dichloromethane

Reaction

Tetracyanoethylene yields a colored π -complex with aromatic compounds; in the case of aromatic amines, phenols and indoles these then react to yield the corresponding tricyanovinyl derivatives [3, 4].



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed for 1 s in the dipping solution or sprayed evenly with the spray solution and dried for a few minutes in a stream of cold air [4, 6] or at 80°C.

Various colored chromatogram zones are formed on a pale yellow background.

Note: For some of the substances the intensities of coloration are only stable for ca. 2 h; in the case of phenols the coloration intensifies during this time [2]. The detection limits for indole derivatives lie in the lower nanogram range.

The reagent can be employed on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Indole and Indole Derivatives [6]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
---------------	-------------------------------------------------------------------------------------

Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Toluene – ethyl acetate (50 + 15).
Migration distance	7.5 cm
Running time	17 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in reagent solution for 1 s and dried in a stream of cold air.

5-Nitroindole (hR_f 40–45), 5-chloroindole (hR_f 60–65) and indole (hR_f 70–75) yielded orange-yellow chromatogram zones on a pale yellow background. If the chromatogram was exposed to ammonia vapor for 15 s the color was intensified. The detection limits were 10 ng substance per chromatogram zone.

In situ quantitation: The photometric scanning in reflectance was carried out at a mean wavelength of $\lambda = 460$ nm ($\lambda_{\max}(5\text{-nitroindole}) = 450$ nm, $\lambda_{\max}(5\text{-chloroindole}) = 460$ nm, $\lambda_{\max}(\text{indole}) = 480$ nm; Fig. 1).

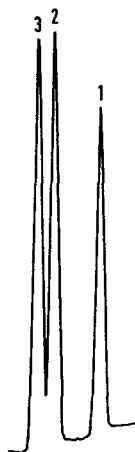


Fig. 1: Reflectance scan of a chromatogram track with 80 ng substance per chromatogram zone: 5-nitroindole (1), 5-chloroindole (2) and indole (3).

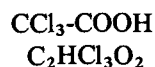
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Trichloroacetic Acid Reagent

Reagent for:

- Steroids [1]
- Alkaloids from
e.g. veratrum [1],
colchicum [2]
- Digitalis glycosides [3, 4]
- Vitamin D₃ [5]
- Benzodiazepin-2-one derivatives [6]



$$M_r = 163.39$$

Preparation of Reagent

Dipping solution	Dissolve 5 g trichloroacetic acid in 50 ml chloroform [2] or ethanol.
Storage	The dipping solution may be kept for at least 1 week at room temperature.
Substances	Trichloroacetic acid Chloroform

Reaction

The reaction mechanism has not yet been elucidated.

Method

After drying in a stream of cold air the chromatograms are immersed for 1 s in the reagent solution or sprayed with it and then heated at 120°C for 10 min.

Mainly light blue fluorescent zones appear under long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: A few drops of 30% hydrogen peroxide solution are added to this reagent when detecting digitalis glycosides [4, 7]. Digitalis glycosides of the A series fluoresce yellow-brown, those of the B series brilliant blue and those of the C series pale blue [7]. The fluorescence can be stabilized and intensified by dipping the plate into a solution of liquid paraffin — *n*-hexane (1 + 2).

The reagent can be employed on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Steroids [1]

Method	Ascending, one-dimensional development in a trough chamber. The layer was conditioned for 30 min at 0% relative humidity after the samples had been applied.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK), which had been prewashed by developing once to the upper edge of the plate with chloroform — methanol (50 + 50) and then activated at 110°C for 30 min.
Mobile phase	Cyclohexane — diethyl ether (50 + 50).
Migration distance	6 cm
Running time	ca. 15 min

Detection and result: The chromatogram was dried in a stream of cold air and then intensively irradiated with UV light ($\lambda = 365 \text{ nm}$) for 2 min and then immersed in the reagent solution for 1 s. It was finally heated to 120°C for 10 min and after cooling dipped into liquid paraffin — *n*-hexane (1 + 2) to intensify and stabilize the fluorescence. Light blue fluorescent zones were produced under long-wavelength UV light ($\lambda = 365 \text{ nm}$) by cholesterol (hR_f 20–25), coprostanol (hR_f 25–30), 4-cholesten-3-one (hR_f 40–45), 5 α -cholestan-3-one (hR_f : 60) and coprostanone (hR_f 70).

In situ quantitation: The fluorimetric analysis was carried out under long-wavelength UV light ($\lambda_{\text{exc}} = 365 \text{ nm}$, $\lambda_{\text{em}} > 430 \text{ nm}$; Fig. 1). The detection limit was several nanograms substance per chromatogram zone.

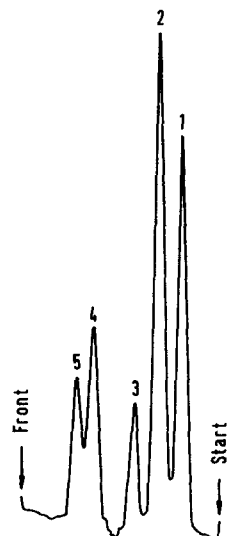


Fig. 1: Fluorescence scan of a chromatogram track with 250 ng of each substance per chromatogram zone (exception coprostanol with 500 ng). Cholesterol (1), coprostanol (2), 4-cholesten-3-one (3), 5 α -cholestan-3-one (4), and coprostanone (5).

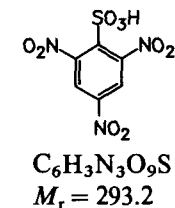
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Trinitrobenzenesulfonic Acid Reagent (TNBS Reagent)

Reagent for:

- Amino acids [1]
- Aminoglycoside antibiotics [2, 3]
e.g. neomycin, gentamycin



Preparation of Reagent

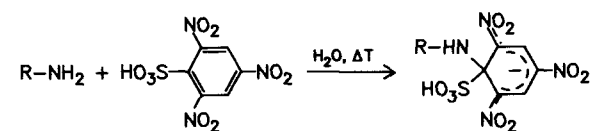
Dipping solution Dissolve 100 mg 2,4,6-trinitrobenzenesulfonic acid in a mixture of 20 ml acetone, 20 ml ethanol and 10 ml water.

Storage The reagent should always be freshly made up.

Substances 2,4,6-Trinitrobenzenesulfonic acid
Acetone
Ethanol

Reaction

On heating primary amines form colored MEISSENHEIMER complexes with trinitrobenzenesulfonic acid.



Method

The chromatograms are freed from mobile phase, immersed in the reagent solution for 1 s or sprayed evenly with it, dried in a stream of warm air and heated to 100°C for 5 min.

Deep yellow-colored zones are formed on a pale yellow background; these remain visible for ca. 2 days.

Note: Ammonia interferes with the reaction and must be removed from the layer completely before application of the reagent.

The reagent can be employed on silica gel and kieselguhr layers. NH_2 layers are not suitable.

Procedure Tested

Gentamycin C Complex [2]

Method	Ascending, one-dimensional development at 10–12°C in a twin-trough chamber with 5 ml ammonia solution (25%) in the second trough; chamber saturation for 15 min.
Layer	HPTLC plates Silica gel 60 (MERCK) which had been pre-washed by developing three times with chloroform – methanol (1 + 1) and then dried at 110°C for 30 min.
Mobile phase	Chloroform – ammonia solution (25%) – ethanol (10 + 10 + 9); the <i>lower organic phase</i> was employed.
Migration distance	ca. 5 cm
Running time	ca. 20 min

Detection and result: The HPTLC plates were freed from mobile phase (ca. 30 min in a stream of cold air, the ammonia must be removed as completely as possible), immersed in the reagent for 1 s, dried in a stream of warm air and then heated to 100°C for 4–5 min.

The gentamycins C_{1a} (hR_f 35–40), C_2/C_{2a} (hR_f 40–45) and C_1 (hR_f 45–50) yielded intense yellow-colored zones on a pale yellow background.

In situ quantitation: The UV absorption was recorded in reflectance at $\lambda = 353$ nm. The detection limit was 40 ng substance per chromatogram zone.

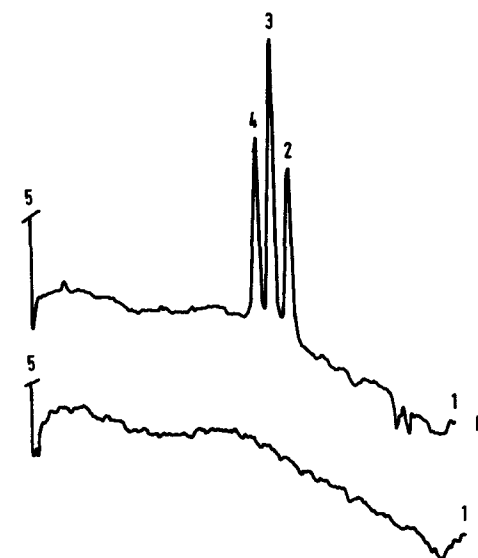


Fig. 1: Reflectance scan of a blank track (A) and of a gentamycin standard track (B) with 800 ng gentamycin mixture per starting zone. Start (1), gentamycin C_{1a} (2), gentamycin C_2/C_{2a} (3), gentamycin C_1 (4) and solvent front (5).

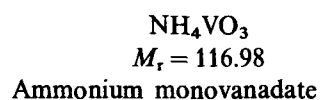
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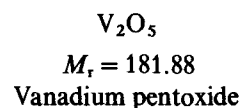
Vanadium(V) – Sulfuric Acid Reagent (Mandelin's Reagent) (Ammonium Monovanadate – Sulfuric Acid or Vanadium Pentoxide – Sulfuric Acid Reagent)

Reagent for:

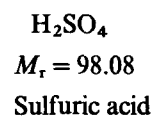
- Carbohydrates and derivatives
e.g. monosaccharides [1–4]
oligosaccharides [1–3]
sugar alcohols (hexitols) [1, 3, 4]
alkylglycosides [1, 3]
uronic acids [1, 3]



- Glycols [1, 4]
- Diethylene glycol [4]
- Reducing carboxylic acids [1]
e.g. ascorbic acid, glycolic acid,
oxalic acid, pyruvic acid



- Steroids [5]
- Antioxidants [5]
- Vitamins [5]
- Phenols [5, 6]
- Aromatic amines [5]
- Antihypertensives (β -blockers) [7, 9]
- Pyrazolidine derivatives [8]
- Laxatives [10]



Preparation of Reagent

Dipping solution Dissolve 0.6 g ammonium monovanadate (ammonium metavanadate) in 22.5 ml water and carefully add 2.5 ml conc. sulfuric acid and 25 ml acetone.

Spray solution Ia Dissolve 1.2 g ammonium monovanadate in 95 ml water and carefully add 5 ml conc. sulfuric acid [1, 2].

Spray solution Ib Dissolve 18.2 g vanadium pentoxide in 300 ml aqueous sodium carbonate solution, ($c_{\text{Na}_2\text{CO}_3} = 1 \text{ mol/l}$), with heating; after cooling carefully add 460 ml sulfuric acid, ($c_{\text{H}_2\text{SO}_4} = 2.5 \text{ mol/l}$), and make up to 1 l with water. Remove excess CO_2 in the ultrasonic bath [1, 3, 4].

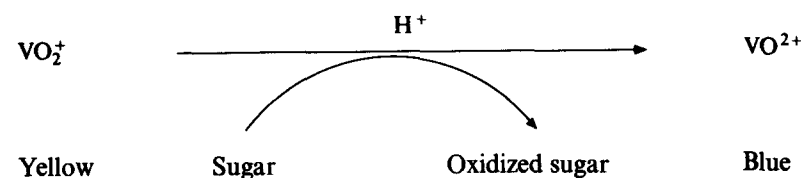
Spray solution II β -Blockers: Saturated solution of ammonium monovanadate in conc. sulfuric acid [7].

Storage The reagent solutions may be stored for an extended period.

Substances Ammonium monovanadate
Vanadium(V) oxide
Sulfuric acid
Sodium carbonate decahydrate
Acetone

Reaction

The yellow colored vanadyl(V) ion is transformed to the blue vanadyl(IV) ion by reaction with reducing agents.



Method

The chromatogram is freed from mobile phase, immersed for 2 s in the dipping solution or sprayed evenly with spray solution Ia or Ib until it starts to appear transparent and then heated to 100–120°C for 5 min. After cooling the chromatogram can be sprayed briefly once more to decolorize the background [4].

Spray solution II is employed for the detection of β -blockers [7]; here the plates are analyzed immediately after spraying.

In the case of carbohydrates blue chromatogram zones are produced on a yellow background that slowly fades [2]. Steroids, vitamins, antioxidants, phenols and aromatic amines yield, sometimes even at room temperature, variously colored chromatogram zones [5]. β -Blockers and laxatives also acquire various colors [7, 10]. The detection limits are in the nanogram to microgram range [5].

Note: The reagent can be employed on silica gel, kieselguhr, Si 50 000 and cellulose layers. At room temperature sugars and sugar derivatives react at different rates depending on the functional groups present [1], e.g. ketoses react more rapidly than aldoses. It is possible to differentiate substance types on this basis [1, 3].

Procedure Tested

β -Blockers [9]

Method	Ascending, one-dimensional development in a HPTLC chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK) which had been pre-washed by developing once with chloroform — methanol (50 + 50) and then heated to 110°C for 30 min.
Mobile phase	Ethyl acetate — methanol — ammonia solution (25%) (40 + 5 + 5).
Migration distance	6 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase (20 min in a stream of warm air), immersed in the dipping solution for 2 s, dried briefly in a stream of warm air and heated to 120°C for 10 min.

Atenolol (hR_f 25–30), bunitrolol (hR_f 40–45) and alprenolol (hR_f 50–55) appeared as light blue to white zones on a yellow background in visible light. They were visible as light blue fluorescent zones on a faint blue background in long-wavelength UV light ($\lambda = 365$ nm).

In situ quantitation: The fluorimetric determination was carried out in UV light ($\lambda_{exc} = 313$ nm, $\lambda_{em} > 390$ nm; Fig. 1). The detection limits were 50 ng substance per chromatogram zone.

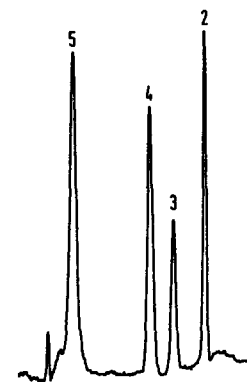


Fig. 1: Fluorescence scan of a chromatogram track with 1 μ g substance per chromatogram zone. Start (1) atenolol (2), bunitrolol (3), alprenolol (4) and "dipping" front (5).

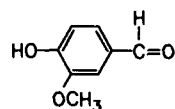
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Vanillin – Phosphoric Acid Reagent

Reagent for:

- Steroids, sterols [1–4]
e.g. estrogens, anabolics [2, 3]
- Triterpenes [4, 5]
- Cucurbitacins
(bitter principles) [4, 6]
- Digitalis glycosides [7, 8]
- Prostaglandins [9]
- Saponins [10, 11]

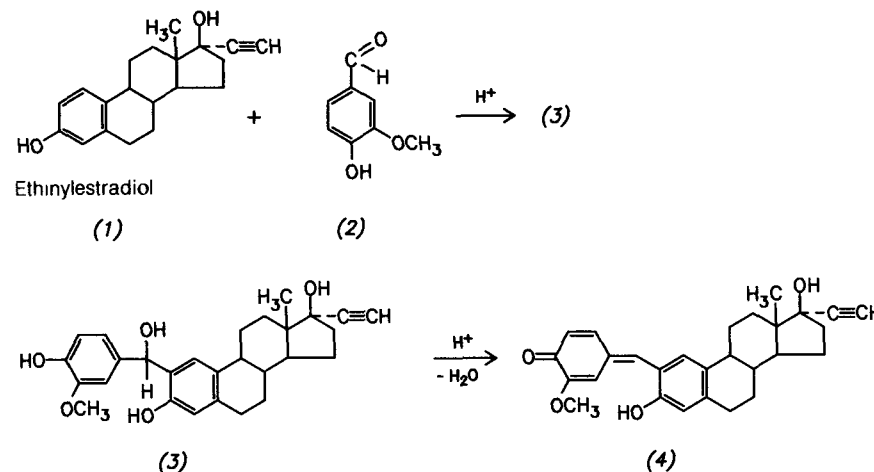
C₈H₈O₃ $M_r = 152.15$

Vanillin

H₃PO₄ $M_r = 98.00$ *ortho*-Phosphoric acid

Reaction

The reaction probably occurs according to the following scheme:



Method

The dried chromatograms (15 min in a stream of warm air) are briefly immersed in the reagent solution or homogeneously sprayed with it and then heated to 120–160°C for 5–15 min. Colored zones are produced on a pale background; in the case of digitalis glycosides, cucurbitacins and sterols these fluoresce in long-wavelength UV light ($\lambda = 365$ nm) [4, 7].

Note: The reagent can be employed on silica gel, kieselguhr, Si 50 000 and RP layers. Hydrochloric or sulfuric acid can be employed in place of phosphoric acid (q.v.). The detection limits for steroids and digitalis glycosides are several nanograms per chromatogram zone.

Preparation of Reagent

Dipping solution Dissolve 1 g vanillin (4-hydroxy-3-methoxybenzaldehyde) in 25 ml ethanol and add 25 ml water and 35 ml *ortho*-phosphoric acid.

Storage The solution may be kept at 4°C for ca. 1 week.

Substances Vanillin
ortho-Phosphoric acid (85%)
Ethanol

Procedure Tested

Estrogens, Anabolics [3]

Method	Ascending, one-dimensional development in a twin-trough chamber with 2 ml 25% ammonia solution in the chamber free from mobile phase. After application of the sample and before development the plate was preconditioned for 60 min over the mobile phase.
Layer	HPTLC plates Silica gel 60 (MERCK), which had been pre-washed three times with chloroform — methanol (50 + 50) and then dried at 110°C for 30 min.
Mobile phase	Chloroform — methanol (19 + 1).
Migration distance	ca. 5 cm
Running time	ca. 10 min

Detection and result: The chromatogram was dried in the air, immersed in the reagent solution for 5 s and then heated to 120°C for 5 min. *cis*-Diethylstilbestrol (hR_f 15–20) and *trans*-diethylstilbestrol (hR_f 40–45) turned red; while ethynylestradiol (hR_f 50–55) appeared blue.

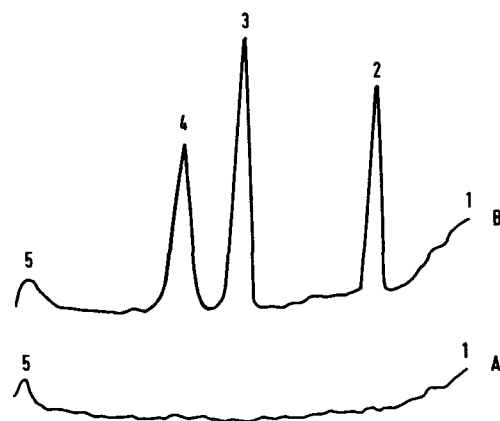


Fig. 1: Absorption scan of a blank track (A) and of a mixture of estrogen anabolics with 100 ng of each substance per chromatogram zone (B). Start (1), *cis*-diethylstilbestrol (2), *trans*-diethylstilbestrol (3), 17- α -ethynylestradiol (4), solvent front (5).

In situ quantitation: The absorption photometric analysis was made at $\lambda = 540$ nm (ethynylestradiol) and $\lambda = 605$ nm (diethylstilbestrol). The detection limit for ethynylestradiol was 12 ng and that for diethylstilbestrol 3 ng per chromatogram zone.

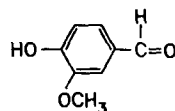
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Vanillin — Potassium Hydroxide Reagent

Reagent for:

- Amines, amino acids
e.g. aminoglycoside
antibiotics [1, 2]



$C_8H_8O_3$
 $M_r = 152.15$
Vanillin

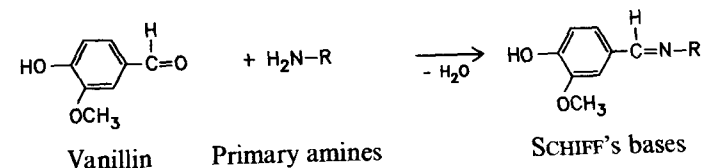
KOH
 $M_r = 56.11$
Potassium
hydroxide

Preparation of Reagent

Dipping solution I	Dissolve 1 g vanillin (4-hydroxy-3-methoxy-benzaldehyde) in 50 ml 2-propanol.
Dipping solution II	Make 1 ml 1 mol/l potassium hydroxide solution up to 100 ml with ethanol.
Storage	The solutions may be stored for several days in the refrigerator.
Substances	Vanillin 2-Propanol Potassium hydroxide solution (1 mol/l) Ethanol

Reaction

Vanillin reacts with primary amines in weakly basic media to form fluorescent or colored SCHIFF's bases whereby colored phenolates are also produced at the same time.



Method

The chromatograms are freed from mobile phase (10 min stream of warm air), immersed for 1 s in solution I, dried for 10 min at 110°C, then immersed in solution II and finally heated once again for 10 min to 110°C. Various colored zones are produced; these can frequently be excited to fluorescence by long-wavelength UV light ($\lambda = 365$ nm) [3].

Note: The dipping solution can also be employed as a spray solution.

Ornithine, proline, hydroxyproline, pipecolic acid and sarcosine yield red zones, glycine greenish-brown and the other amino acids weakly brown ones [3]. The colors of the zones are different if an alcoholic solution of potassium carbonate is used for basification instead of dipping solution II.

Characteristic fluorescence often appears under long-wavelength UV light ($\lambda = 365$ nm) after drying for the first time (before the use of reagent solution II): e.g. ornithine yields a strong greenish-yellow fluorescence and lysine a weak one, while hydroxyproline appears light blue.

The reagent can be employed on silica gel, kieselguhr or cellulose layers. Amino phases and polyamide layers are unsuitable.

Procedure Tested

Gentamycin C Complex [1, 2]

Method

Ascending, one-dimensional development at 10–12°C in a twin-trough chamber with 5 ml ammonia solution (25%) in the second part of the chamber; chamber saturation for 15 min.

Layer	HPTLC plates Silica gel 60 (MERCK), which had been pre-washed by developing three times with chloroform — methanol (1 + 1) and heated to 110°C for 30 min after each development.
Mobile phase	Chloroform — ammonia solution (25%) — ethanol (10 + 10 + 9); the <i>lower organic phase</i> was employed.
Migration distance	ca. 5 cm
Running time	ca. 20 min

Detection and result: The chromatogram was freed from mobile phase (45 min, stream of cold air), immersed in reagent solution I for 1 s, dried at 110°C for 10 min, immersed in solution II and heated to 110°C for a further 10 min. Gentamycins C_{1a} (hR_f 35–40), C_2/C_{2a} (hR_f 40–45), C_1 (hR_f 45–50) produce yellow zones on a pale yellow background.

In situ quantitation: The absorption photometric determination was carried out in long-wavelength UV light ($\lambda = 392$ nm). The detection limit was 40 ng gentamycin per chromatogram zone (Fig. 1).

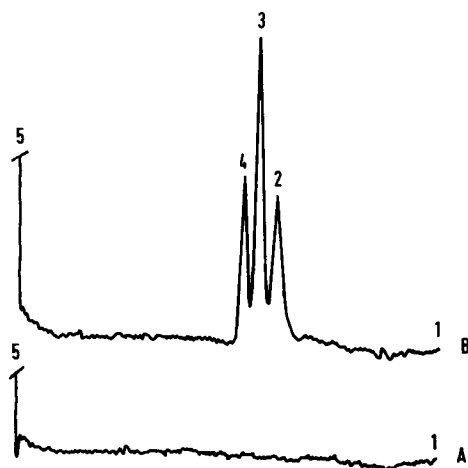


Fig. 1: Absorption scan of a blank track (A) and of a gentamycin standard track (B) with 500 ng gentamycin $C_1 - C_{2a}$ mixture per starting zone. Start (1), gentamycin C_{1a} (2), gentamycin C_2/C_{2a} (3), gentamycin C_1 (4) and solvent front (5).

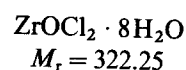
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Zirconium(IV) Oxide Chloride Reagent

Reagent for:

- Flavonoids [1–3]
- Mycotoxins
e.g. sterigmatocystine [2]
- Steroids
e.g. estrogens [4, 7]
- Purines, pyrimidines [4]
- Cardiac glycosides [5]
- Lipids
e.g. fatty acids, phospholipids, cholesterol,
cholesteryl esters [4]
triglycerides [4, 6]
- Sugars [4]
- Prostaglandins [4]



Preparation of Reagent

Dipping solution	Dissolve 1 g zirconium(IV) oxide chloride octahydrate (zirconyl chloride) in 50 ml methanol.
Spray solution	Dissolve 2 to 25 g zirconium(IV) oxide chloride octahydrate in 100 ml methanol [1], ethanol – water (55 + 15) [5] or water [4].
Storage	The reagent solutions may be stored for an extended period.
Substances	Zirconium(IV) oxide chloride octahydrate Methanol Ethanol

Reaction

The reaction mechanism has not yet been elucidated [4].

Method

The chromatograms are freed from mobile phase, immersed in the dipping solution for 1 s or sprayed evenly with it and normally heated to 100–120°C for 10 min [2, 5, 6], or sometimes to 150–180°C for 5 to 60 min [4].

Mainly yellow-green to bluish fluorescent chromatogram zones are formed on a dark background under long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: Flavonoids react with the reagent even at room temperature [1]; mycotoxins, steroids, purines, pyrimidines, cardiac glycosides and lipids only react on heating [2, 4–6]. Zirconyl sulfate can be used to replace the zirconyl chloride in the reagent; this is reported to result in an increase in the sensitivity to certain groups of substances (e.g. cholesteryl esters, triglycerides) [4].

The chromatogram can be sprayed with liquid paraffin – *n*-hexane (2 + 1) to increase the fluorescence intensity [2]. The detection limits per chromatogram zone are 0.5–1 ng for cardiac glycosides [5], triglycerides [6] and sterigmatocystine [2] and 25 pg for estrogens [7].

The reagent, which can also be employed to impregnate the layer before chromatography, is best suited for silica gel layers [4]; it can, however, also be employed on aluminium oxide, kieselguhr, Si 50000, cellulose and polyamide layers [4].

Procedure Tested

Estriol, Estradiol, Estrone [7]

Method

Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK), which had been pre-washed by developing once with chloroform – methanol (1 + 1) and then activated at 110°C for 30 min before sample application.
Mobile phase	Toluene – ethanol (9 + 1).
Migration distance	7 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air, conditioned for 15 min in ammonia vapor (placed in a twin-trough chamber whose second part contained 25% ammonia solution). It was then immediately immersed in the reagent solution for 1 s and heated to 180°C for 15 min.

Estriol (hR_f 10–15), estradiol (hR_f 30–35) and estrone hR_f 40–45) appeared under long-wavelength UV light ($\lambda = 365$ nm) as light blue fluorescing chromatogram zones on a dark background.

In situ quantitation: Fluorimetric analysis was carried out by excitation at $\lambda_{exc} = 313$ nm and detection at $\lambda_{fl} > 390$ nm. The detection limits were 25 pg substance per chromatogram zone (Fig. 1).

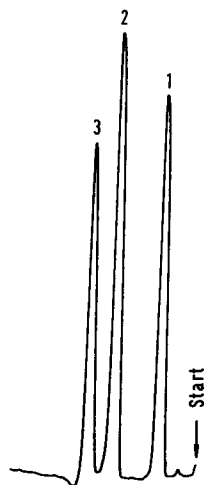


Fig. 1: Fluorescence scan of a chromatogram of ca. 50 ng each of estriol (1), estradiol (2) and estrone (3) per chromatogram zone.

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Name Reagents Reagent Acronyms

Name Reagents

BESTHORN's Reagent	347 – 350
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CARR-PRICE Reagent	206 – 209
EMERSON REAGENT	151 – 153
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NEU's Flavone Reagent	277 – 280
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Reagent Acronyms

ANS-Reagent	191 – 194
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Foreword

Chromatographic methods often develop in a cyclic manner: The discovery of a new separation technique naturally stimulates interest concerning the method; attention wanes when another technique appears on the horizon and soon interest is directed at the new technique. There is then a confrontation between the old methods and a critical comparison of the advantages and disadvantages of the new methods. This sometimes leads to a renaissance of the older method, which has then been the subject of further development in the meantime. In this context discoveries made in connection with the modern technique are often used to advantage for the older one.

This is what happened in liquid chromatography, as it was influenced by the instrumentation developed for gas chromatography. A similar process has occurred in thin-layer chromatography. It has experienced a new impetus during the last 10 years as a result of instrumentation and automation together with the availability of improved stationary phases and working techniques. Nevertheless, one of the great advantages of thin-layer chromatography is that it provides a wealth of information rapidly and economically without the necessity for expensive equipment. The large numbers of publications are a proof of this popularity: According to Sherwood, 3800 articles were published during the years 1990–1991 in which thin-layer chromatography was used to separate mixtures of substances, for identification and purification or in conjunction with quantitation. Thus TLC/HPTLC is a standard analytical method today. The applications are far more numerous than the publications. This results in the method frequently not being described in detail in the literature. There it often says tersely: “the identification or the determination was carried out by means of planar chromatography”.

Thin-layer chromatography is a separation technique: Emphasis is laid on the possibility of separating substances and characterizing them, initially based on their mobility in a system of two phases. The components are then detected. Earlier this was only done by chemical reactions on the layer or by the measurement of absorption or fluorescence in short- or long-wavelength light. Later the palette of possibilities was enlarged so that thin-layer chromatography now possesses a wide variety of detection methods. This is the great advantage of the method compared to column techniques (HPLC, CZE, GC). The rational choice from numerous general, selective or specific detection methods provides a wealth of information concerning the structure of the substance being analysed, which culminates in

greatly enhanced probability of the identification of the separated substance. All this is achieved relatively simply and very cheaply with the sensitivity of the method often equalling that of HPLC.

For these reasons great emphasis has been placed, from the very beginning, on detection in planar techniques. First compilations on this subject can be found in our monograph on paper chromatography. Methods for 221 detection reagents and advice on their proper use were described forty years ago. These reagents were then modified for thin-layer chromatography by Waldi in 1962 and by Wimmer, Heusser and Krebs in 1966 and collected in the already classical monograph by Egon Stahl. Zweig and Sherma enlarged the collection ten years later. It also appeared in the Merck company brochure „Anfärbereagenzien für die Dünnschicht- und Papier-Chromatographie“. Unfortunately little attention was paid in the later literature to the important combination of physical separation and chemical detection. It is only in recent years that efforts have been made to develop more sensitive detection reagents to improve the selectivity and increase the precision of the quantitation that follows.

It is therefore very much to be welcomed that the four authors — all specialists in the field of thin-layer chromatography — have devoted themselves to the production of a monograph covering this complex of topics. This assignment is no mean task, but it is as current as ever. The planned, detailed description in 5 volumes has no parallel in the world literature. It can only be attempted by colleagues who have many years of personal experience of thin-layer chromatography and have lovingly accompanied the development of the method for over 35 years with their own research. The methods described in this book are so clearly set out that they can be followed without recourse to the original literature. In addition the interested worker will also find a wealth of literature references, to serve as a basis for personal study. The authors are to be congratulated on their achievement. It is to be hoped that this monograph will not only ease routine work in the laboratory but will also act as a stimulus for the further development and growth of thin-layer chromatography.

Prague, September 1993

Karel Macek

Preface to Volume 1b

This volume is the second of a series of practice-orientated TLC/HPTLC books published in excellent quality by VCH Publishers. As in the first volume, a selection of reagents and detection methods have been reviewed with the intention of helping the practical analyst increase the detection specificity of routine samples separated by thin-layer chromatography.

This volume is divided into two parts which encompass about the same amount of material as Volume 1a. Thus Part I begins with specific detection methods including the known photochemical, thermochemical and electrochemical activation methods. Here microchemical reactions are described that are carried out with the use of reagents. Detection involves the use of light, heat and electric current.

Then follows a selection of group-specific reagents, in response to requests from practical workers after the publication of Volume 1a. This part should be seen in reference to the monographs that follow or have already been published.

The section on "Reagent Series" has also been included at the request of practical workers. There are many publications describing the sequential application of a series of different reagents to the same chromatogram. Reagents intended for preparatory reaction of certain substances so that the final reagent applied will yield specific detection results are dealt with in this volume. Independent reagents, each capable of detection, combined on the same chromatogram to increase selectivity, e.g. by specifically altering certain colors (= potentiated multi-detection) will be treated in Volume 1c. Such combinations are frequently used in the field of clinical and forensic chemistry and in the analysis of natural product extracts.

Part I, which contains tested examples together with more than 220 literature references, is followed by Part II; this consists of 65 reagent monographs in alphabetical order. Once again, each includes an example that has been tested in the laboratory and is supplemented by numerous literature references. In the past it is just these references that have helped provide the practical worker with an entry to earlier literature.

Great importance has also been attached to the inclusion of photographs of original chromatograms in the examples tested along with absorption photometric or fluorimetric scans. These show at a glance that modern thin-layer chromatography is a microanalytical separation method that should be taken seriously and that its development certainly conforms to the state of the analytical art. Those of us more senior in years will immediately recognize the advances made in the method over the last decade.

Particular attention has been devoted to the compilation of the cumulative index. Every reference work is only as good as its indexing system. For this reason a presentation has been chosen which allows one to recognize immediately in which volume the key word appears. The same also applies to named reagents which can be traced back to the original publication in almost all cases in order to be able to correct any errors that have crept in. This type of presentation will be continued in future volumes.

This volume includes a new feature in the collection of reagents into groups that are discussed comparatively. Such groups include the chloramine T, the diaminobenzaldehyde or the vanillin reagents. The iodine reagents are also dealt with as a group. We have not yet been able to make a similar comparison of the Dragendorff or the ninhydrin reagents. They will follow in Volume 1c.

The fact that our treatment of group-specific reagents is still incomplete illustrates the enormous amount of work involved in compiling a reagent series of value to the practical worker. Those who have also been confronted with such a task appreciate our difficulties. Thanks and recognition are due to J. Ganz, I. Klein and Bl. Meiers and to S. Netz for their tireless work. Thanks are also expressed to the numerous undergraduate and graduate students who have assisted in checking the derivatization reactions, together with E. Otto, G. Schon and Dipl. Ing. M. Heiligenthal in whose capable hands lay the technical preparation of this book.

Prof. Dr. H.-J. Kallmayer (University of Saarland) and Dr. H.-O. Kalinowski (Giessen University) provided generous assistance in the formulation and interpretation of often difficult reaction paths. We had always wished such a cooperation, and it proved to be of great benefit to the resulting work.

We also thank Baron, J.T. Baker, Camag, Desaga, Macherey-Nagel, Merck and Riedel de Haën for their generous support of the experimental work. The monographs would never have been written without their aid.

Our especial thanks are due to the ladies of VCH Publishers, Mrs. Banerjee-Schulz, Dr. Dyllick and Mrs. Littmann for the way they have converted our ideas for the design and layout of this book into reality and for the pleasant cooperation over the past four years.

In spite of all our efforts and careful work errors are bound to remain. We would appreciate our readers sending us their suggestions for improvements. The positive reaction we received to Volume 1a gave us enormous pleasure and has provided us with the motivation to continue our work on the series.

Saarbrücken, Gießen and Darmstadt, December 1992

Hellmut Jork
Werner Funk
Walter Fischer
Hans Wimmer

Preface to Volume 1a

This book is the result of cooperation between four colleagues, who have been working in the field of thin-layer chromatography for many years and, in particular, took an active part in the development from hand-coated TLC plate to commercially available precoated plates and instrumental thin-layer chromatography. This development was accompanied by improvements in the field of detection of the separated zones. In particular, it became necessary to be able to do this with ever decreasing quantities of substance, so that the compilation "Anfängerreagenzien" by E. Merck, that had been available as a brochure for many, many years, no longer represented the state of the art of thin-layer chromatography.

It was against this background and in view of the fact that there is at present no contemporary monograph on thin-layer chromatography that this book was produced. It is intended as an introduction to the method, a reference book, a laboratory handbook in one, i.e., far more than just a "Reagent Book".

The first part of the book consists of a detailed treatment of the fundamentals of thin-layer chromatography, and of measurement techniques and apparatus for the qualitative and quantitative evaluation of thin-layer chromatograms. In particular, the prechromatographic derivatization techniques used to improve the selectivity of the separation, to increase the sensitivity of detection, and to enhance the precision of the subsequent quantitative analysis are summarized in numerous tables.

Particular attention has been devoted to the fluorescence methods, which are now of such topicality, and to methods of increasing and stabilizing fluorescence emissions. Nowhere else in the literature is there so much detailed information to be found as in the first part of this book, whose more than 1000 literature references may serve to stimulate the reader to enlarge his or her knowledge.

Nor has a general introduction to the microchemical postchromatographic reactions been omitted: it makes up the second part of the book.

This second part with its 80 worked-through and checked detection methods forms the foundation of a collection of reagent reports (monographs), which can be extended to several volumes and which is also sure to be welcomed by workers who carry out derivatizations in the fields of electrophoresis and high-pressure liquid chromatography. Alongside details of the reagents required and their handling and storage, the individual reports also contain details about the reaction conditions.

Wherever possible, dipping reagents have been employed instead of the spray reagents that were formerly commonplace. These make it easier to avoid contaminating the laboratory, because the coating of the chromatogram with the reagent takes place with less environmental pollution and lower health risks; furthermore, it is more homogeneous, which results in higher precision in quantitative analyses.

It is possible that the solvents suggested will not be compatible with all the substances detectable with a particular reagent, for instance, because the chromatographically separated substances or their reaction products are too soluble. Therefore, it should be checked in each case whether it is possible to employ the conditions suggested without modification. We have done this in each report for one chosen class of substance by working through an example for ourselves and have documented the results in the "Procedure Tested"; this includes not only the exact chromatographic conditions but also details concerning quantitation and the detection limits actually found. Other observations are included as "Notes". Various types of adsorbent have been included in these investigations and their applicability is also reported. If an adsorbent is not mentioned it only means that we did not check the application of the reagent to that type of layer and not that the reagent cannot be employed on that layer.

Since, in general, the reagent report includes at least one reference covering each substance or class of substances, it is possible to use Part II of this book with its ca. 750 references as a source for TLC applications. Only rarely are earlier references (prior to 1960), which were of importance for the development of the reagent, cited here.

There is no need to emphasize that many helpful hands are required in the compilation of such a review. Our particular thanks are due to Mrs. E. Kany, Mrs. I. Klein, and Mrs. S. Netz together with Dipl.-Ing. M. Heiligenthal for their conscientious execution of the practical work.

We would also like to thank the graduate and postgraduate students who helped to check the derivatization reactions and Mrs. U. Enderlein, Mrs. E. Otto, and Mrs. H. Roth, whose capable hands took care of the technical preparations for the book and the production of the manuscript. We would particularly like to thank Dr. Kalinowski (Univ. Gießen) for his magnificent help in the formulation of the reaction paths for the reagent reports. Our thanks are also due to Dr. F. Hampson and Mrs. J. A. Hampson for translating the German edition of the book into English.

We thank the Baron, J. T. Baker, Camag, Desaga, Macherey-Nagel and E. Merck companies for their generous support of the experimental work.

Our particular thanks are also due to Dr. H. F. Ebel and his colleagues at VCH Verlagsgesellschaft for the realization of our concepts and for the design and presentation of the book and for the fact that this work has appeared in such a short time.

In spite of all our care and efforts we are bound to have made mistakes. For this reason we would like to ask TLC specialists to communicate to us any errors and any suggestions they may have for improving later volumes.

Saarbrücken, Gießen and Darmstadt, October 1989

Hellmut Jorl
Werner Funk
Walter Fisch
Hans Wimm

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Introduction

Modern thin-layer chromatography is a microanalytical separation method whose importance has been increasing steadily since the 1970s [1]. UNGER has spoken of the renaissance of the 30-year-old liquid chromatographic method [2] and MAJORS postulated a positive continuation of the development on the basis of a poll of experts [3]; this development has been confirmed in a review of organic analysis in the year 1990 [4]. KELKER writes that the former “poor man’s chromatography” remains practically irreplaceable and is used at the bench in almost every single organic chemical/synthetic/research laboratory [5].

The currently most important fields of application of thin-layer chromatography can be seen in Fig. 1. The proportion of publications in the fields of pharmacy and environmental analysis has increased over that in previous years. There has also been an appreciable increase in the fields of clinical and forensic chemistry and in biochemistry.

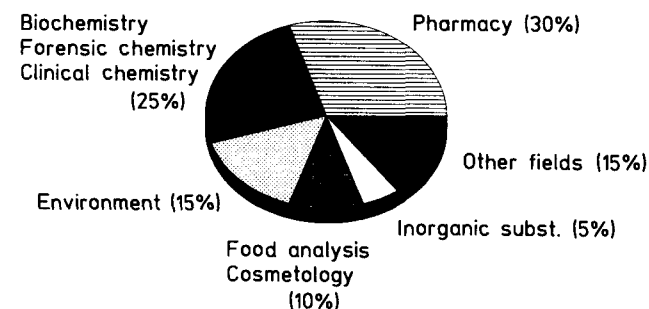


Fig. 1: Fields of application of thin-layer chromatography (TLC/HPTLC) during the period 1988–1991.

The reason for this lies not least in the increasing instrumentalization and deliberate automation of all those processes which were earlier particularly subject to error (Fig. 2). Modern high performance thin-layer chromatography (HPTLC) is no longer inferior to other liquid chromatographic techniques with respect to precision and sensitivity (Fig. 3) [6].

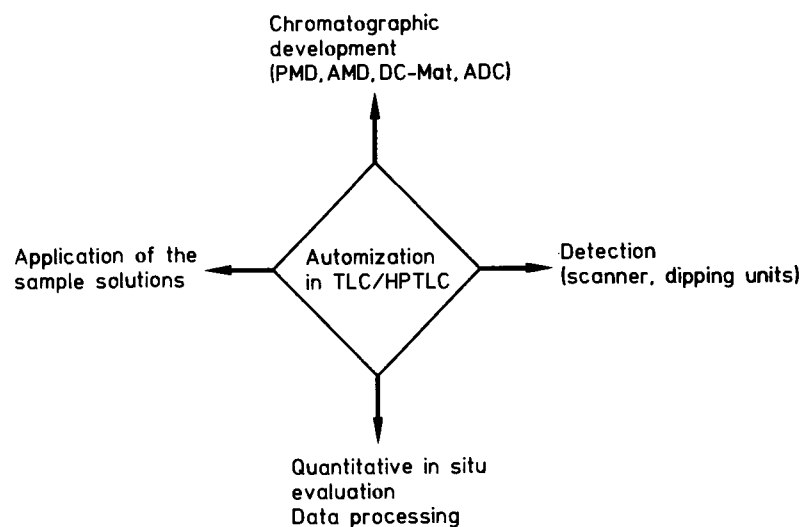


Fig. 2: The steps in the process of thin-layer chromatography that have been instrumentalized and automated to a large degree in the recent past. PMD = Programmed Multiple Development, AMD = Automated Multiple Development, DC-Mat or ADC = Automatic Development Chamber.

The development of methods of coupling TLC with other chromatographic methods and with physical methods of measurement has brought enormous advantages. The first attempts to couple gas chromatography on-line with thin-layer chromatography were made by NIGAM [7], JANAK [8-10] and KAISER [11]. VAN DUK [12] described the on-line coupling of column chromatography with thin-layer chromatography as early as 1969. He divided the eluent stream with a splitting system and demonstrated afterwards that at least three different components could be detected thin-layer chromatographically in an apparently uniform fraction of column eluate.

Today 80-90% of all HPLC separations are carried out on RP phases, while silica gel layers are used for more than 90% of all thin-layer chromatography. This provides the possibility of coupling different separation mechanisms together.

Separation by adsorption chromatography takes place preferentially as a result of hydrogen bonding or dipole-dipole interactions. Hence, separation of mixtures of substances on silica gel layers by lipophilic solvents primarily takes place according to polarity differences. Further separation within a polarity group can then be achieved either two-dimensionally or off-line by partition chromatography on another TLC plate (Fig. 4).

Analysis methods	Detection limit per liter of water				
	mg	µg	ng	pg	fg
Spectrophotometry	10	1	0.1	0.01	0.001
Fluorimetry	10	1	0.1	0.01	0.001
HPTLC	10	1	0.1	0.01	0.001
HPLC (DAD)	10	1	0.1	0.01	0.001
GC (ECD, NPD)	10	1	0.1	0.01	0.001
Inverse voltammetry	10	1	0.1	0.01	0.001
Radioimmunoassay	10	1	0.1	0.01	0.001
Mass spectroscopy	10	1	0.1	0.01	0.001
Laser fluorescence spectroscopy	10	1	0.1	0.01	0.001
	ppm	ppb	ppt	ppq	
Concentration					

Fig. 3: Sensitivity of various methods of determination.

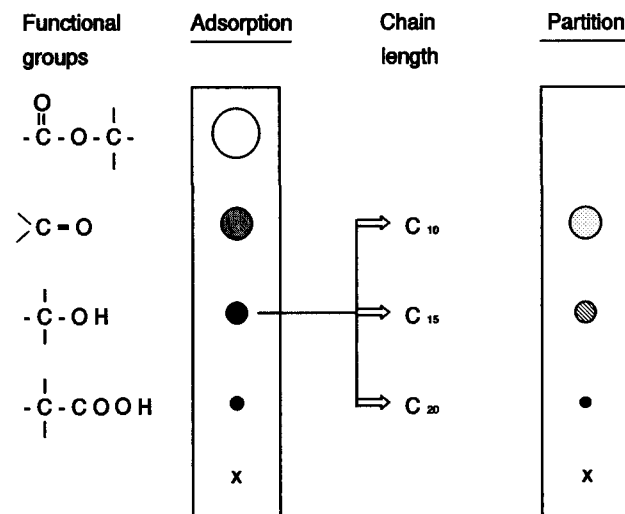


Fig. 4: Coupling the separation principles of adsorption and partition chromatography.

For the same reason it is also possible to use Over Pressure Layer Chromatography (OPLC) on-line for prefractionation or as a clean-up method for HPLC [13, 14]: A group separation according to polarity is followed by a differentiation of the substances according to their differing lipophilicities (Fig. 5).

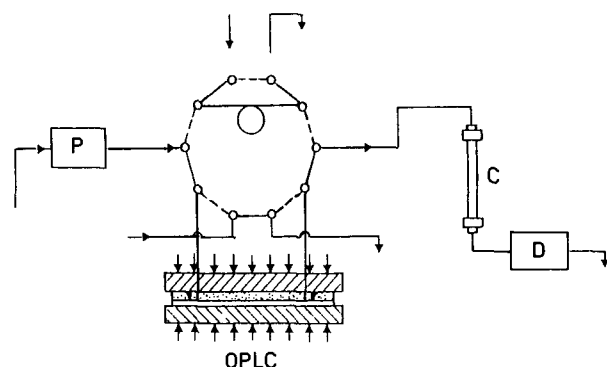


Fig. 5: Schematic representation of OPLC-HPLC coupling; P = pump system, C = column, D = detector.

Conversely successful on-line coupling of HPLC to TLC is also possible. HOFSTRAAT [15–17] and BAHEYNS and LING[18] have described suitable apparatus. BURGER, for instance, was able to demonstrate that adsorptive separation of selective cut fractions of an HPLC eluate from RP partition chromatography could be separated into up to 700 individual peaks [19, 20]. Here the thin-layer chromatographic step employed the Automated Multiple Development (AMD) technique. These investigations and the results of KROKER, FUNK and EISENBEISS [21, 22] demonstrate the enormous power of such on-line coupling techniques in a very impressive manner.

In their investigations of caramel MÜLLER et al. [23, 24] demonstrated that such combinations can also be applied to purely aqueous fractions of column eluates: A column-chromatographic separation was made on TSK gels according to hydrophobic interactions, the eluates of individual peaks were then led directly into an on-line sample preparator (OSP 2) equipped with small Polyspher® RP 18 CAT cartridges (OSP 2) [25, 26], in which the organic components were enriched (Fig. 6). A brief rinsing and drying process was followed by elution with a little organic solvent and on-line application of the eluate to silica gel 60 HPTLC plates using the Linomat C (Fig. 7). This was followed by thin-layer chromatographic separation and detection of the individual components.

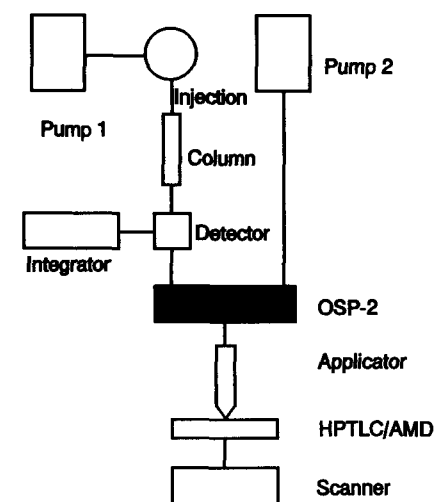


Fig. 6: Schematic representation of HPLC-HPTLC coupling by means of the OSP-2 sys (MERCK) for "post-column enrichment" of the column eluate fractions.

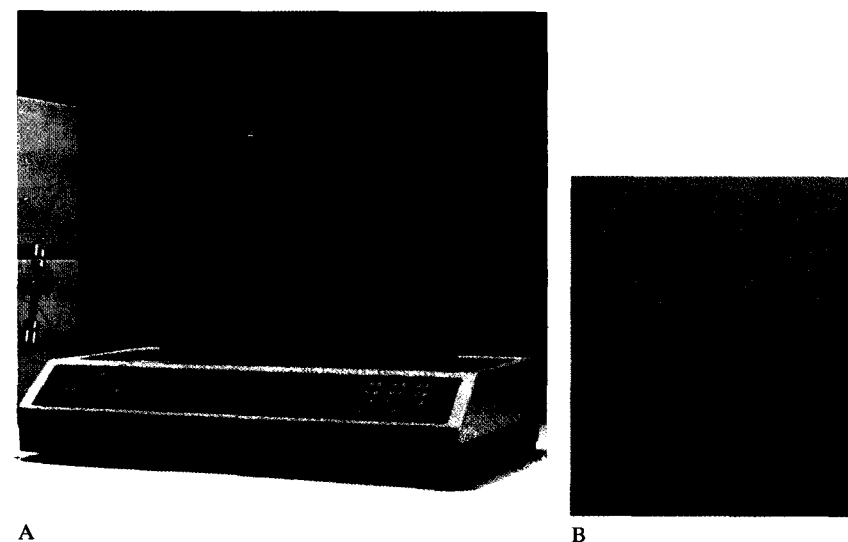


Fig. 7: Linomat C (CAMA) for on-line transfer of column eluate fractions to TLC/HPTLC p (A) and application scheme (B).

The on-line principle has also been extended into the field of detection (Fig. 8). Thus, it is now possible to record FTIR [27–31] and Raman spectra in situ [32, 33], and there have been considerable advances in the on-line coupling of thin-layer chromatography with mass spectrometry. Here it has been, above all, the research groups of WILSON [34–36] and BUSCH [37–40] that have made the necessary instrumental and methodological advances, so that TLC must no longer be viewed as merely a clean-up method. Rather it forms the essential central point for all these on-line coupling techniques.

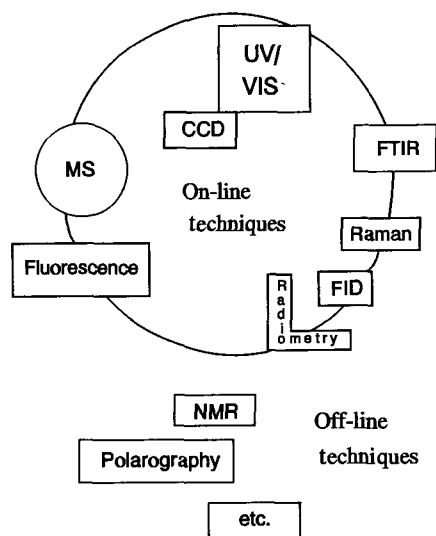


Fig. 8: Possibilities for on-line coupling of thin-layer chromatography with physical measurement and determination methods. CCD = Charge Coupled Device Detection.

The range of microchemical pre- and postchromatographic derivatization methods has also been enlarged, for instance photo- and thermochemical methods have yielded unexpected results. Group-specific reagents have been refined and new ones suggested. Reagent series are receiving greater attention and more sensitive reagents have been developed. These have led logically to the organization of this volume.

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Part I

Specific Detection Methods

1 Activation Reactions

Every reaction chain is only as strong as its weakest link. It was LIEBIG who illustrate this truism with a barrel (Fig. 9): The shortest stave determines how high the barrel can be filled.

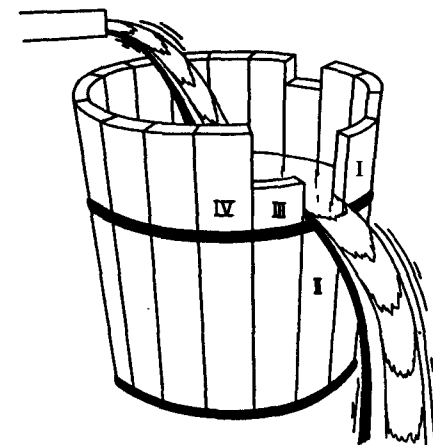


Fig. 9: LIEBIG's barrel.

This principle also applies to chromatography. For instance, the best of separation methods is of no avail if the results of the separation cannot be detected.

It is well known that the sorbents used in thin-layer chromatography possess large specific surface areas [1], that can interact with the substances being separated. This applies to the time taken for development and naturally when the chromatogram has been dried. The additional effect of energy (heat, visible light, UV, X-rays, γ -radiation, high potential) can be used to bring about desired photo- and thermochemical reactions. The following are among the phenomena that have been observed:

- dehydrogenations and dehydrations
- the formation of oxidation products in the presence of oxygen that are readily detected by the SRS technique (separation – reaction – separation)
- rearrangement of *trans* to *cis* compounds
- production of ions and radicals which then react further e.g. in chain reactions, yield stable higher molecular weight substances that can be colored and/or can emit fluorescent light
- pyrolysis phenomena [2].

The inorganic sorbents act as catalysts in all this [3, 4]. The pH also probably plays a role. Reactions that do not otherwise occur are observed on acid silica gel [5] or basic aluminium oxide layers. Reactions of this type have also been observed for amino [6–8] and RP phases [9]. The products of reaction are usually fluorescent and can normally be used for quantitative analysis since the reactions are reproducible.

Such reactions can be promoted by exposing the chromatogram to the vapors of hydrogen halides, to nitric acid fumes [4], to ammonia or oxides of nitrogen [2] in suitable reaction chambers [10]. Ammonium hydrogen carbonate, first proposed by SEGURA and GOTTO is also suitable [11].

Impregnation with ammonium acetate or ammonium hydrogen sulfate serves the same purpose [11–13]. In conjunction with the TLC separation previously carried out it is even possible to obtain group-specific and sometimes substance-specific information.

The reactions discussed in the next section are those carried out without any application or impregnation with reagent solutions or exposure to reagent vapors.

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1.1 Photochemical Activation

It was observed relatively early that chemically labile compounds — such as vitamin carotenes — decompose, either on application to the TLC layer or during the TI separation that follows. This phenomenon was primarily ascribed to the presence of oxygen (oxidation) and exposure to light (photochemical reaction) in the presence of active sorbents, which were assumed to exert a catalytic effect (photocatalytic reaction).

Today all automatic sample applicators blanket the plate with nitrogen; firstly this has the effect that the applied starting zones dry quickly and secondly serves to prevent oxidation of the applied substances.

Some application instruments possess light-absorbent covers to prevent or reduce the action of UV and visible light (Fig. 10).

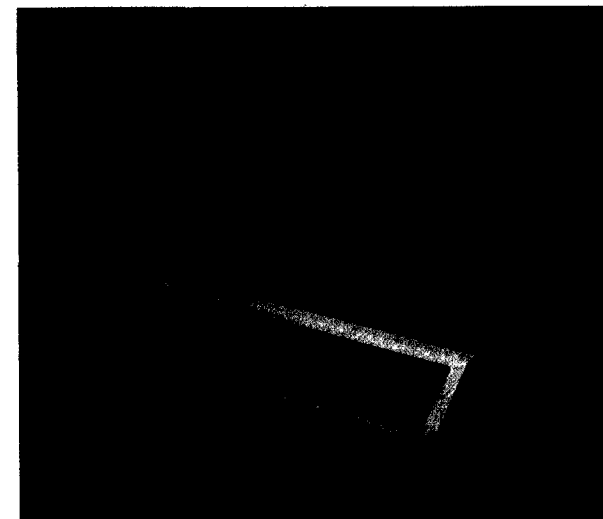


Fig. 10: Linomat IV with protective gas atmosphere (CAMAG).

It has been recommended that the outer walls of the separation chamber be covered with black foil or that the work be carried out in a dark room under green or red light.

More recent chromatogram chambers — e.g. the AMD system (Fig. 11) — only possess a small observation window and this can, if necessary, be covered with a black cloth. Development in the DC-Mat (Fig. 12) or the ADC (Fig. 13) automatic development chambers is carried out entirely in the dark.

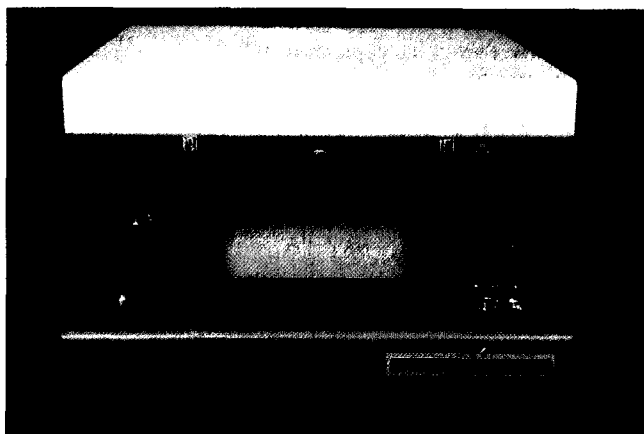


Fig. 11: AMD system (CAMAG).



Fig. 12: DC-Mat (BARON, DESAGA), opened. The “flap” bearing the HPTLC plate is folded upwards during chromatography, so that the development can take place in the dark.



Fig. 13: Automatic development chamber (ADC chamber, CAMAG).

In general photochemical reactions only occur when the affected substance absorbs radiation, i.e. when π or n electrons are raised to an excited π^* state. Interaction of the electric light vector with the electronic shell of the molecule brings about a change in the structure of the electronic shell. This change takes place during one period of oscillation of the light (ca. 10^{-15} s). The altered structure of the electronic shell corresponds to a higher energy state of the molecule. Hence, the molecule is in an electronically excited state [2] This excited singlet state S only has a short life. Excess oscillation energy is immediately conducted away (Fig. 14). Activated molecules return to the ground state once again, whereby one of the following processes can occur [3]:

- Energy rich π^* electrons experience a spin reversal so that the molecule involved passes from the singlet to the corresponding triplet state (Fig. 14, 15/I).
- The excited molecule passes instantaneously from the singlet to the ground state S , with the emission of light (fluorescence) (Fig. 14, 15/II).
- The excess energy of excited molecules is transferred, by collision, to acceptor molecules, which are converted to an excited state while the initially excited molecules return to the ground state (Fig. 15/III).

- The excited singlet or triplet state returns to the ground state by a radiationless deactivation process (Fig. 15/IV).
- A chemical process occurs involving the formation of a new substance with corresponding energetic ground state N (Fig. 14, 15/V). For instance, on aluminium oxide or silica gel layers in the presence of oxygen, anthracene initially yields anthraquinone, that is then oxidized further to yield 1,2-dihydroxyanthraquinone [4, 5]. Alizarin and chrysazin are also formed depending on the properties of the aluminium oxide used [6].
- Starting from the energy level of the triplet state a further spin reversal leads to the ground state S_0 (phosphorescence radiation, Fig. 14, 15/VI).

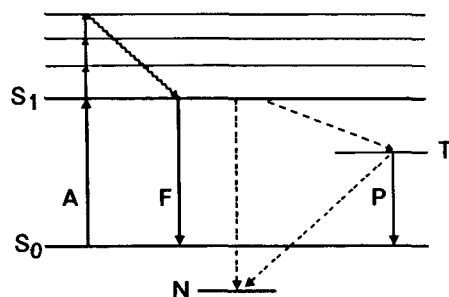


Fig. 14: Schematic representation of the electronic transitions of photochemically excited substances S_0 = ground state, S_1 = first excited singlet state, T = "forbidden" triplet transition, N = ground state of a newly formed compound, A = absorption, F = fluorescence, P = phosphorescence.

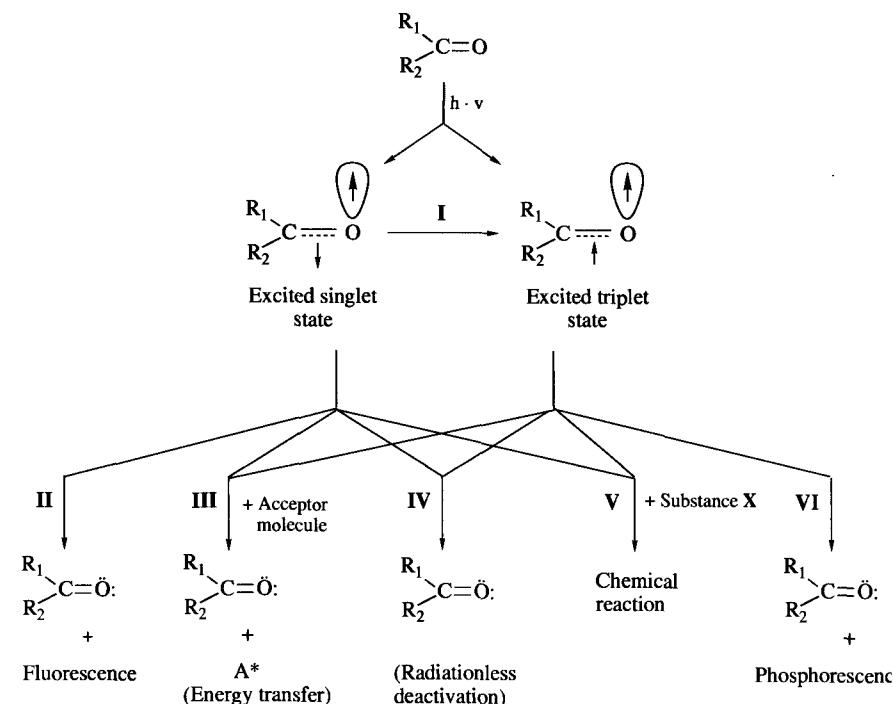


Fig. 15: Possibilities for photochemically induced reactions, using a carbonyl compound as example. $\downarrow\downarrow$ and $\uparrow\uparrow$ = electron spins parallel and antiparallel respectively.

This short discussion should provide an indication of the versatility of photochemical reactions. For example it is possible to synthesize, in a simple manner, complicated ring systems that are difficult to produce by conventional synthetic methods. For these reasons it is only rarely possible to make unequivocal predictions concerning the chemical structures of the products formed particularly if oxygen is present during the course of the reaction.

It is often possible to detect such photochemical reactions with the aid of the SR technique (separation – reaction – separation) [1, 7]. An initial thin-layer chromatographic separation is followed by irradiation of the chromatogram. The irradiated chromatogram is then developed perpendicular to the first direction of development using the same mobile phase. In the absence of any reaction all the chromatogram zones lie on a diagonal. However, if reaction has occurred, the R_f values of the affected substances are displaced into the regions above or below the diagonal during the second development.

STAHL, for instance, was able to demonstrate that on irradiation with long-wavelength UV light the naturally occurring contact insecticides pyrethrin I and II, cinerin I and II and jasmolin I and II present in *Chrysanthemum cinerariifolium* are converted to inactive pyrethrin oxides by the incorporation of oxygen [7].

UV irradiation of piperine, the most important hot substance of pepper, does not lead to the incorporation of atmospheric oxygen [8]. The all-*trans* compound is converted to the *cis-trans* isomer, this can be seen in the chromatogram above the all-*trans* piperine (Fig. 16).

In this case the excited molecules produced on interaction with radiation undergo spin reversal to yield a triplet state with a much longer lifetime than that of the singlet excited state. One or more π -bonds are broken in the triplet state since one of the π -electrons affected is in an antibonding π^* molecular orbital. This means that the σ -bond is free to rotate and *cis* and *trans* isomers can be formed next to each other on recombination of the double bond.

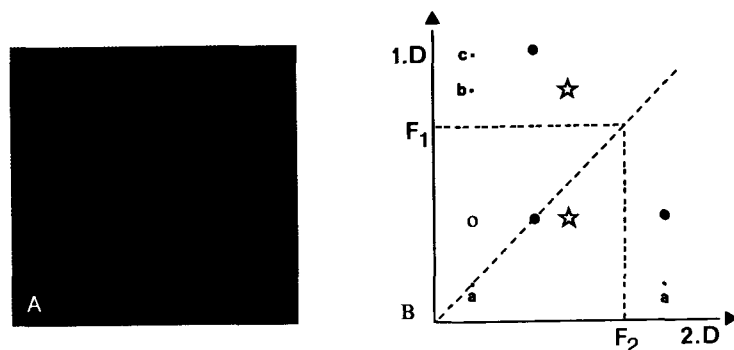


Fig. 16: Detection of *cis/trans* isomerization of piperine by the SRS technique after UV irradiation: (A) original chromatogram, (B) schematic representation.

F_1 , F_2 = mobile phase front after development in the first and in the second dimension; a, b, c = positions of application of the *trans/trans*-piperine before the first (1D) and before the second development 2D; ☆ = *cis/trans*-piperine, ● = *trans/trans*-piperine, ○ = position of the *trans/trans*-piperine after the first development. Irradiation of the chromatogram with long-wavelength UV light after application of *trans/trans*-piperine to position b after the first development (position c was not irradiated!).

SCHUNACK and ROCHELMAYER have described such a *cis/trans* isomerization of annuloline, a weakly basic alkaloid from *Lolium multiflorum* LAM [9]. Irradiation with UV light after the first TLC development simultaneously causes a *cis*→*trans* and a *trans*→*cis* isomerization, so that the SRS technique yields four blue fluorescent

chromatogram zones ($\lambda_{exc} = 365$ nm, $\lambda_{fl} = 422$ nm) at the corners of a rectangle. Detailed investigations carried out in the complete absence of light revealed that the plant produces exclusively *trans*-annuloline and that only this is fluorescent. Hence there are evidently four blue fluorescent spots on the SRS chromatogram because *trans*→*cis* isomerization occurs during work-up of the plant extract and application of the sample solution and *cis*→*trans* and *trans*→*cis* isomerizations occur simultaneously during the UV irradiation after the first TLC development.

Similar processes occur with azo compounds [10]. *trans*-Dimethylaminoazobenzene (butter yellow) yields some of the *cis* isomer on irradiation with long-wavelength UV light and this possesses a lower R_f than the *trans* compound on rechromatography with the same mobile phase using the two-dimensional SRS technique (Fig. 17). IR and MS measurements were used to confirm that no oxygen had been incorporated into the molecule.

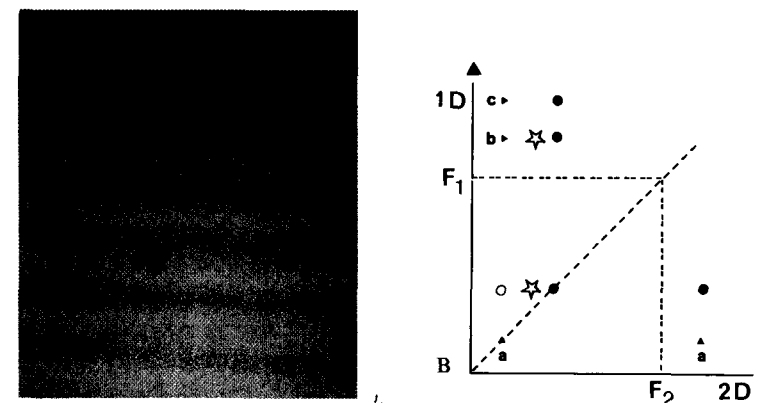
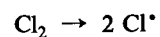


Fig. 17: Detection of the photochemical *cis/trans* isomerization of butter yellow after UV irradiation by using the SRS technique. (A) original chromatogram — treated with hydrochloric acid vapor for better recognition (yellow then turns red) — and (B) schematic representation.

F_1 , F_2 = mobile phase front after development in the first and in the second dimension; a, b, c = positions of application of the *trans*-butter yellow before the first (1D) and before the second development (2D); ☆ = *cis*-butter yellow, ● = *trans*-butter yellow, ○ = position of the *trans*-butter yellow after the first development. Irradiation of the chromatogram with long-wavelength UV light after application of *trans*-butter yellow to position b after the first development (position c was not irradiated!). In contrast to Figure 16 the photochemically produced reaction product lies below the starting compound.

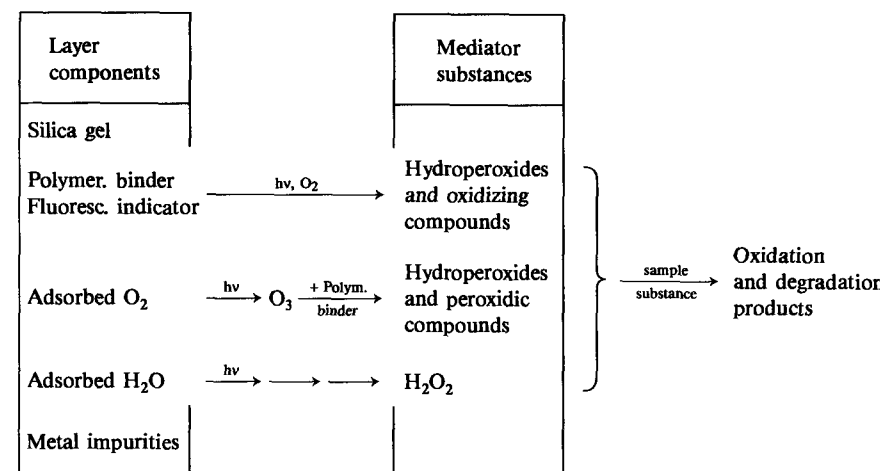
These few examples illustrate impressively how a range of substances can undergo chemical reaction when they are exposed to light while on the TLC plate:

- Wavelengths of about $\lambda = 200$ nm excite olefinic structures in particular, causing $\pi \rightarrow \pi^*$ transitions.
- Wavelengths between $\lambda = 280$ nm and 290 nm or longer are absorbed by carbonyl compounds. Here the free n-electrons of the oxygen enter the antibonding π^* molecular orbital.
- It is possible for homolysis to occur when the light energy absorbed by a molecule reaches or exceeds the bonding energy. Radicals are formed or, in the case of halogens, atomic halogen:



Further characteristic assignments of substance structures to wavelength ranges that are absorbable are to be found in the specialist literature [2, 11–14]. The publications of the research groups of FASSLER [15, 16] and OELKRUG [17–19] reveal that the sorbent can exert a considerable additional effect.

TAKÁCS et al. [27] have also studied the effects of sorbents. They demonstrated that the irradiation of the sorbent layers before use (“activation”) causes changes to occur in the stationary phase chemically altering the chromatographic behavior of 3,5-pyrazolidindione derivatives. The authors attributed these “memory” effects to photochemical oxidation of the binders and other materials. According to the following scheme (p. 21) the water film of the layer yields hydroperoxide and the oxygen ozone, these two then react – possibly under the influence of metallic impurities or fluorescence indicators – with the acrylate and methacrylate polymers. In this manner transmitter substances are produced that greatly increase the reactivity of the layer and which remain active for days. This “post-photo effect” generally leads to the same reaction product as that produced by direct irradiation of the plate after application or after chromatography of the sample under investigation.



FRÜNS has demonstrated this possibility with reserpine and rescinnamine by radiating at the start zone for two hours and obtaining a characteristic zone pattern (fingerprint) after TLC separation of the photochemically produced derivatives [2]. HUCK and DWORCAK exposed developed chromatograms with vanilmandelic acid a homovanillic acid zones to diffuse daylight and observed the formation of fluorescent metabolites that were suitable for direct quantitative analysis [21].

FUNK et al. have used a low-pressure mercury lamp without filter to liberate inorganic tin ions from thin-layer chromatographically separated organotin compounds; they were then reacted with 3-hydroxyflavone to yield blue fluorescent chromatogram zones on a yellow fluorescent background [22]. Quantitative analysis was also possible ($\lambda_{\text{exc}} = 405$ nm, $\lambda_{\text{fl}} = 436$ nm, monochromatic filter). After treatment of a chromatogram with Triton X-100 (fluorescence amplification by a factor of 5) the detection limits for various organotin compounds were between 200 and 500 (calculated as tin).

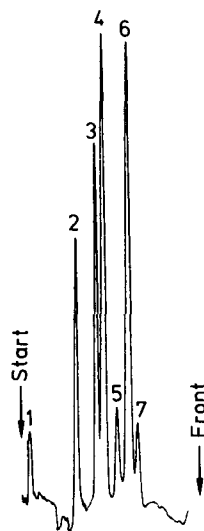
Fifteen β -blockers have also been activated photochemically with the same radiation unit (HERAEUS, Hanau; OSRAM STE 501; UV lamp TNN 15-3200/721)[23]. The detection limits, the working range and associated standard deviation of the methods are listed in Table 1 below. The blue fluorescence of the chromatogram zones ($\lambda_{\text{exc}} = 313$ nm, $\lambda_{\text{fl}} > 390$ nm) was measured after dipping the chromatogram in liquid paraffin - *n*-hexane (1+2). Figure 18 illustrates the separation of seven β -blockers.

The reactions described above also explain reactions that occasionally occur during TLC and are frequently regarded as interferences. Of course, they can also be deliberately employed for photochemical activation of applied or thin-layer chromatographically separated samples.

Table 1: Detection limits, working ranges and method standard deviation V_{xo} for quantitative analysis of β -blockers.

Substance	Detection limit [ng/chromatogram zone]	Working range	V_{xo} [%]
Acebutolol	5	8–70	± 3.7
Atenolol	100	n. a. *)	n. a. *)
Bupranolol	50	60–220	± 2.3
Carazolol	5	10–80	± 4.5
Nadolol	50	n. a. *)	n. a. *)
Pindolol	5	10–90	± 1.7

*) not available

**Fig. 18:** Fluorescence scan of a chromatogram track with 250 ng each of atenolol (1), acebutolol (2), cartelol [3], pindolol (4), bunitrolol (5), alprenolol (6) and penbutolol (7) per chromatogram zone.

While FUNK et al. did not use temperatures above 30 °C during the irradiation times discussed above, SISTOVARIS combined UV irradiation with simultaneous heating (70 °C, 2 h) of the TLC layers [24]. After this treatment nomifensine and its metabolites appeared as intense yellow fluorescent chromatogram zones on a dark background.

This chapter ends with a tested procedure to represent the many photochemical reactions on silica gel.

Procedure Tested

Chelidonine in the Greater Celandine [25]

Layer: Silica gel

Irradiation: $\lambda = 254$ nm

Reaction

The reaction mechanism has not been elucidated. The processes occurring are presumably those already discussed in Section 1.1.

Sample preparation: Dried greater celandine was pulverized and briefly boiled with 0.05 mol sulfuric acid. After cooling to room temperature the mixture was placed in a separating funnel and adjusted to pH 10 with ammonia solution and extracted with chloroform. The organic phase was dried with sodium sulfate and evaporated to dryness under reduced pressure. The residue was taken up in methanol and used as the sample solution for TLC.

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation and the exclusion of light.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK), before application of the samples the layer was developed to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110 °C for 30 min.
Mobile phase	Toluene – methanol (90+10)

Migration distance 7 cm

Running time 15–20 min

Detection and result: The developed chromatogram was dried for 15 min in a stream of warm air and then examined under long-wavelength UV light ($\lambda = 365$ nm):

A whole range of separated celandine extract components are visible as intensely fluorescent chromatogram zones; however, chelidone does not emit fluorescent light at this stage, but fluorescence quenching is likely to occur under short-wavelength UV light ($\lambda = 254$ nm) (Fig. 1A and 1B).

The chromatogram was then irradiated with short-wavelength UV light ($\lambda = 254$ nm) for 3–5 min and examined again under long-wavelength UV light ($\lambda = 365$ nm).

Now chelidone produced an intense green fluorescent chromatogram zone; in addition there were other intensely fluorescent zones in the track of the celandine extracts – some of which were not previously visible or had another color shade (Fig. 1C). In addition the general fluorescence was increased as a result of the UV irradiation. Figure 1 illustrates the corresponding fluorescence scans.

In situ quantitation: The fluorimetric scan was carried out at $\lambda_{\text{exc}} = 313$ nm and the fluorescence emission was measured at $\lambda_{\text{fl}} > 400$ nm (cut off filter) (Fig. 11).

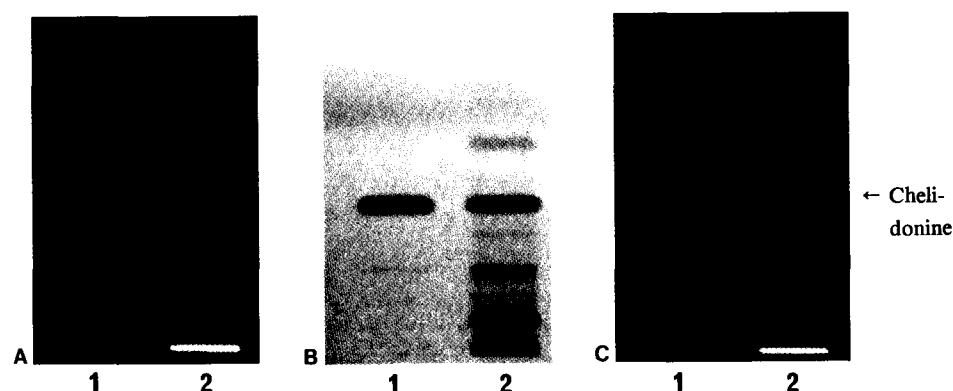


Fig. 1: Chromatogram of celandine extract (Track 2) and a chelidone standard (Track 1): (A) detection of fluorescent zones in long-wavelength UV light, (B) detection of UV absorbing zones in short-wavelength UV light by fluorescence quenching and (C) detection of photochemically activated chromatogram zones after irradiation of the chromatogram with short-wavelength UV light.

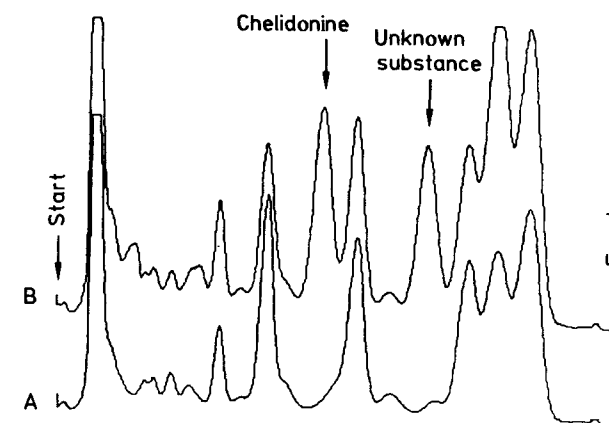


Fig. 11: Fluorescence scan of a *Chelidonium* extract chromatogram track with ca 5 μg chelidone (A) before and (B) after 1 h irradiation with short-wavelength UV light; two new zones are apparent that were not previously detected [25, 26].

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1.2 Thermochemical Activation

As is well known chemical reactions are accelerated by increasing the temperature. This also applies to heterogeneously catalyzed reactions taking place on the surface of porous sorbents such as aluminium oxide or silica gel (Tables 2.1 and 2.2). Such reactions have also been reported on the moderately polar NH_2 layers. ALPERIN et al. have described the activation of cellulose to yield specific information concerning the substances chromatographed [1].

In the simplest case the developed chromatograms are heated to the required temperature on a hot plate (Fig. 19) or in a drying cupboard. More rarely infrared heaters are used to heat the system [2]. Gas chromatograph ovens can be used if no adjustment of the temperature is required [3].

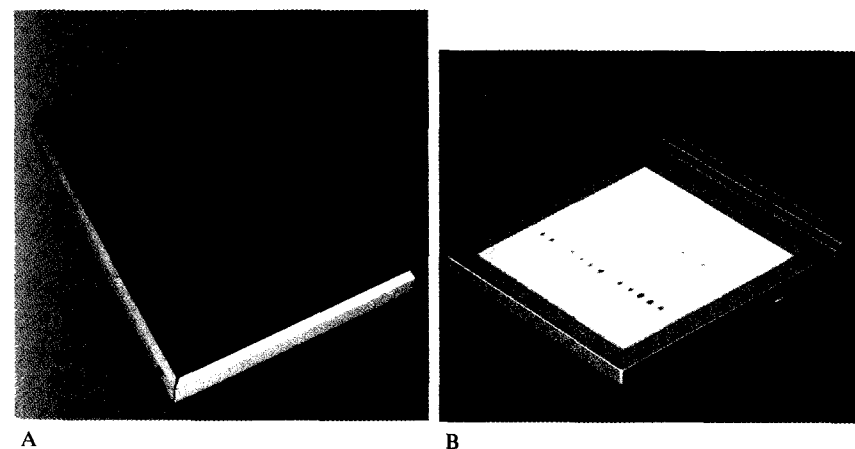


Fig. 19: TLC plate heater III (CAMAG) (A), (DESAGA) (B).

When the compounds are heated close to their decomposition temperatures, in contact with the surface of the active sorbents, while fluorescent substances are produced. Further heating can, however, lead to complete carbonization. The details of the reactions taking place are not currently known [4].

SEGURA and GORTO have postulated that nitrogen-containing compounds form derivatives of the type R-N=CH-CH=CH-NH-R , in a similar manner to the reaction

of malonaldehyde with amino acids to yield SCHIFF's bases — a hypothesis that is supported by the occurrence of appropriate IR bands [5].

In general compounds with heteroatoms (N, O, S and P) are more amenable to “fluorescence reactions” than pure hydrocarbons. Under the influence of the catalytic sorbents substances rich in π -electrons are formed, that conjugate to “rigid” reaction products that are fluorescent when appropriately excited. The formation of fluorescent derivatives is frequently encouraged by gassing with nitrogen or carbon dioxide.

Changes of pH can also yield specific evidence. Thus, it is frequently possible to alter the excitation and fluorescent wavelengths of many fluorescing compounds in this manner. In addition there is a range of nonfluorescent substances that can be derivatized by exposure to ammonia gas, ammonium hydrogen carbonate or acids (e.g. HCl, HBr) to yield products that are able to fluoresce. The impregnation of the layer with ammonium acetate or hydrogen sulfate, that is frequently recommended, serves the same purpose. Examples of this behavior are to be found in the reagent monographs.

The following Tables 2.1 to 2.3 summarize some examples based exclusively on thermochemical reactions on the sorbent surface which lead to the formation of fluorescent reaction products. The derivatives formed frequently remain stable for weeks [6] and the fluorescence can frequently be intensified and/or be stabilized by treatment with viscous liquids (liquid paraffin, Triton X-100, polyethylene glycol etc.).

Quantitation is possible in many cases [6–15]. However, the activation reaction does not always yield a single reaction product (check by SRS method!), so the dependence of the linear response interval on temperature and duration of heating must be checked for each product. It can be taken as a rule of thumb that there will be a linear response between measurement signal and amount applied over the range 10 to 100 ng substance per chromatogram zone [5].

Since the literature cited did not reveal a significant effect of the differing pore systems of the various types of layer the aluminium oxide and silica gel types (60, 80, 100, etc.) are not specified. The same applies to binders, fluorescence indicators and trace impurities in the sorbents.

Table 2.1: Summary of some examples of detection after merely heating aluminium oxide layers (Types 150/T or 60/E) after chromatography.

Substances	Temperature/time	Remarks	R
Pesticides, e.g. aminocarb, captan, difolatan, landrin, rotenone	200 °C, 45 min	Induction of fluorescence in weakly fluorescent or nonfluorescent pesticides and amplification of natural fluorescence. There are some differences between basic and acidic aluminium oxide layers.	[
Δ^4 -3-Ketosteroids, e.g. testosterone and <i>epi</i> -testosterone in urine	180 °C, 20 min	Pale blue induced fluorescence ($\lambda_{fl} = 440$ nm) for Δ^4 -3-ketosteroids, detection limit: 5 ng.	
Δ^4 -3-Ketosteroids, e.g. trimethylsilyl-testosterone	180 °C, 20 min or 150 °C, 20 min	Conversion of Δ^4 -3-ketosteroids or their trimethylsilyl or acetyl derivatives in fluorescent components, whereby the detection limits were improved by 65% for the acetates. Δ^5 -3-keto- and Δ^5 -3-OH-steroids also react with the same sensitivity.	[
Testosterone	180 °C, 20 min	Induced fluorescence ($\lambda_{fl} > 430$ nm, cut off filter) by thermal treatment of the chromatogram, the fluorescence increased by a factor of 2.5 by dipping in a solution of Triton X-100 — chloroform (1+4). Working range: 2–50 ng substance per chromatogram zone. Prewashing the layers with methanol-ammonia solution (25%) (50+50) increased the precision.	[
Testosterone	180 °C, 20 min	Induced fluorescence and fluorescence amplification by a factor of 25 by dipping the chromatogram in a solution of Triton X-100 — chloroform (1+4).	
Δ^4 -3-Ketosteroids, e.g. progesterone in plasma	150 °C, 20 min	Conversion of Δ^4 -3-ketosteroids into fluorescent derivatives ($\lambda_{fl} = 440$ nm). Relatively selective for progesterone at 150 °C, detection limit: 2–5 ng.	

Procedure Tested

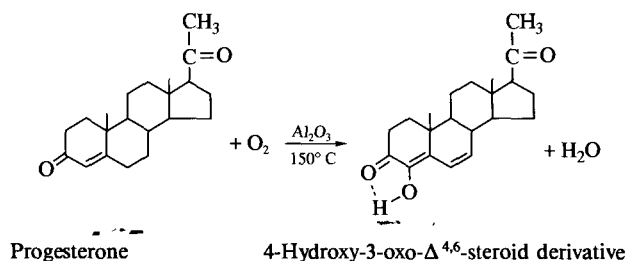
Testosterone [9, 15]

Layer: Aluminium oxide

Temperature: 180 °C

Reaction

At elevated temperatures in the presence of oxygen the aluminium oxide layer catalyzes the formation of blue fluorescent “aluminium oxide surface compounds” with 4-hydroxy-3-oxo- $\Delta^{4,6}$ -steroid structures [4]. Aluminium oxide acts as an oxidation catalyst for an activated methylene group.



Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates Aluminium oxide 150 F ₂₅₄ (MERCK); before application of the samples the layer was developed twice to its upper edge with methanol – ammonia solution (25 %) (50 + 50) to pre-cleanse it and then dried after each development at 120 °C for 30 min.
Mobile phase	Toluene – 2-propanol (10+1)
Migration distance	8 cm
Running time	25 min

1.2 Thermocnemical Activation

Detection and result: The dried chromatogram was heated in the drying oven at 180 °C for 20 min. After cooling to room temperature it was dipped twice for 1 s into a solution of Triton X-100 – chloroform (1+4) which stabilized the fluorescence and increased its intensity by a factor of 2.5. Between the two dipping steps the chromatogram was air-dried in the dark for 30 min until the chloroform had completely evaporated.

Testosterone (R_f : 65 – 70) appeared under long-wavelength UV light ($\lambda = 365$ nm) as a pale blue fluorescent zone on a dark background.

The detection limit was less than 2 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric scan was carried out at $\lambda_{\text{exc}} = 365$ nm and fluorescence emission was measured at $\lambda_{\text{fl}} > 430$ nm (cut off filter Fl 43, Fig. 1).

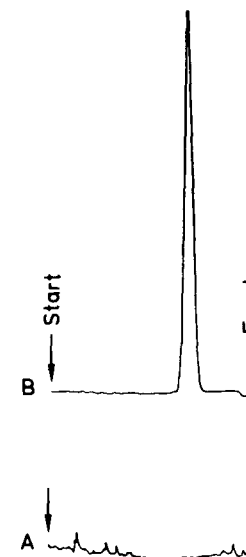


Fig. 1: Fluorescence scan of a blank track (A) and of a chromatogram track with 4 ng testosterone (B).

Analogous examples have been described for “silica gel chromatograms”. Table 1 gives an overview.

Table 2.2: Summary of some examples of fluorimetric detection after merely heating silica gel layers after chromatography.

Substances	Temperature/time	Remarks	Ref.
Essential oil components	800–900°C	Induction of fluorescence in a special apparatus.	[18]
Steroids, e.g. cholesterol, triolein, androsterone; sugars, e.g. fructose, glucose, ribose; amino acids, pyrimidines, purines, alkaloids	110–150°C, 2–12 h	Conversion to fluorescent derivatives by heating.	[5]
Alkaloids, e.g. raubasine and its metabolites in plasma, urine and bile	120°C, 1 h	Amplification of the natural fluorescence of raubasine ($\lambda_{fl} = 482$ nm), detection limit 20 ng.	[10]
Alkaloids, e.g. reserpine, rescinnamine	105°C, 2 h	Induced fluorescence ($\lambda_{fl} > 500$ nm, cut off filter). Possibly formation of 3-dehydro derivatives.	[19]
Alkaloids, e.g. reserpine, ajmaline, rescinnamine	105°C, 2 h or 105°C, 15 h	Induction of stable fluorescence ($\lambda_{fl} > 480$ nm, cut off filter), detection limits 5–20 ng.	[12]
Alkaloids, e.g. cocaine, ecgonine, benzoylecgonine, ecgonine methyl ester	280°C, 8 min or 260°C, 10–30 min	Pale blue induced fluorescence ($\lambda_{fl} > 390$ nm, cut off filter), fluorescence amplification by a factor of 2 on dipping in liquid paraffin solution; detection limits: < 10 ng.	[13]
Alkaloids, e.g. lupanine, angustifoline, sparteine, lupinine, hydroxylupanine	130°C, 17–35 h	Induced blue fluorescence ($\lambda_{fl} = 400$ nm), detection limits: 10 ng.	[6]
Pesticides, e.g. dursban, azinphos-methyl, menazon, imidan, phosalone, zinophos	200–225°C, 20–120 min	Induced fluorescence or amplification of natural fluorescence; detection limits: 10–300 ng.	[20]
Organophosphorus pesticides, e.g. coumaphos, menazon, maretin, dursban	200°C, 45 min	Induced fluorescence or amplification of natural fluorescence, detection limits: 1–80 ng.	[21]

Table 2.2: (continued)

Substances	Temperature/time	Remarks	Ref.
Pesticides, e.g. fuberidazol	200°C, 45 min	Amplification of the natural fluorescence of some pesticides and bathochromic shift of the excitation and emission maxima; detection limits: 5–100 ng.	[2]
Pesticides, e.g. coumatetralyl, methabenz-thiazuron, propylisom, naptalam, thioquinox, warfarin etc.	200°C, 45 min	Induced fluorescence ($\lambda_{fl} > 430$ nm, cut off filter); detection limits: 6–600 ng.	[2]
Coumaphos	200°C, 20 min	Residue analysis; induced fluorescence on heating ($\lambda_{fl} > 400$ nm); detection limit: 1 ng.	[1]
Potasan, coumaphos, coroxon	200°C, 20 min	Induced blue fluorescence ($\lambda_{fl} = 430$ nm or 450 nm), identification of the fluorescent derivatives as chlorferon or 4-methylumbelliferone.	[1]
Coumaphos	200°C, 20 min	Residue determination in honey, induced fluorescence ($\lambda_{fl} > 400$ nm, cut off filter); detection limit: 0.5 ng.	[1]
Rubratoxin B	200°C, 10 min	Induced fluorescence that can be intensified by gassing the previously heated chromatogram plates with ammonia vapors (10 min). This also alters the color of the emitted light to pale blue.	[1]
Glucose or methylglucosides	135°C, 3 min or 140°C, 10 min	Induced yellow fluorescence.	[1]
Sugar derivatives	“Mild heating over a Bunsen burner”	No details of whether fluorescence was produced or if a carbonization reaction occurred.	[1]
Sugars, e.g. glucose, fructose, galactose, mannose etc.	160°C, 10 min	Production of fluorescence by heating the chromatogram after covering it with a glass plate. Sugar alcohols and C ₁ -C ₁ bonded oligosaccharides do not react; detection limit: 10 ng.	[1]

Table 2.2: (continued)

Substances	Temperature/time	Remarks	Ref.
Sugars, e.g. glucose, glucosamine, fucose, raffinose, cellobiose, methylated sugars	80 → 260°C, gradient or 200°C, 5 min	Production of fluorescence by temperature gradients (10°C/30 s) to determine the optimum heating temperature for the individual substances. Oligosaccharides require higher temperatures than monosaccharides. Detection limit: 1 nMol. The fluorescence colors are characteristic particularly for the methylated sugars.	[3]
Lipids, e.g. β -sitosterol, geraniol, dolichol, squalene, cholesterol	200°C, 15 min	Induced fluorescence; detection limits: <1 μ g cholesterol.	[3]
C-Nucleosides	Moderate heating on a hot plate	No details of whether fluorescence or carbonization was produced.	[29]
Nomifensine and metabolites	70°C, 2 h + UV ₂₅₄	Heating and simultaneous UV irradiation produced intense yellow fluorescence ($\lambda_{\text{fl}} > 460$ nm, cut off filter).	[30]

Procedure Tested

Tropane Alkaloids [35, 36]

Cocaine, ecgonine, ecgonine methyl ester, benzoylecgonine

Layer: Silica gel

Temperature: 260°C

Reaction

At elevated temperatures and possibly under the catalytic influence of the sorbent surface there is probably elimination of functional groups to yield aromatic ring systems that are excited to fluorescence under long-wavelength UV light ($\lambda = 365$ nm).

Method

Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer

HPTLC plates Silica gel 60 F₂₅₄ (MERCK) .

Mobile phase

Methanol – water – dioxane – sodium acetate solution (aqueous, 0.2 mol/L, pH 8.0) (60+28+12+10)

Migration distance

5 cm

Running time

30 min

Detection and result: The chromatogram was briefly dried in a stream of cold air then heated for 10 to 30 min at 260°C in a drying oven. After cooling to room temperature (ca. 15 min) it was dipped in a solution of liquid paraffin – *n*-hexane (1 + 2) for 1 min. This stabilized the fluorescence and intensified it by a factor of about 2.

On examination under long-wavelength UV light ($\lambda = 365$ nm) ecgonine methyl ester (hR_f 30–35), cocaine (hR_f 45–50), ecgonine (hR_f 55–60) and benzoylecgonine (hR_f 70–75) appeared as pale blue fluorescent chromatogram zones on a dark background.

The detection limits were less than 10 ng substance per chromatogram zone.

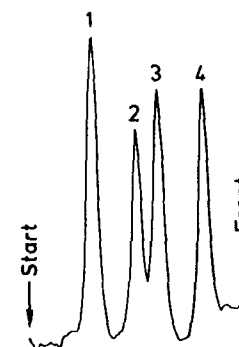


Fig. 1: Fluorescence scan of a chromatogram zone with 300 ng each of ecgonine methyl ester (1), cocaine (2), ecgonine (3) and benzoylecgonine (4) per chromatogram zone.

Note: The sodium acetate was added to the mobile phase solely to improve the separation. It had no detectable effect on the production of fluorescence during thermal activation, since the fluorescence reaction also occurred in the absence of sodium acetate.

In situ quantitation: The fluorimetric scan was carried out at $\lambda_{\text{exc}} = 313 \text{ nm}$ and the fluorescence emission was measured at $\lambda_{\text{fl}} > 390 \text{ nm}$ (cut off filter Fl 39) (Fig. I).

The last example for thermal activation to be discussed involves amino phases. Table 2.3 lists the publications concerning the specific detection of sugars and creatine derivatives by means of the fluorescence obtained on heating mobile phase-free "amino layer chromatograms".

Table 2.3: Summary of some examples of fluorimetric detection after thermal treatment of amino layers after chromatography.

Substances	Temperature/time	Remarks	Ref.
Sugars, e.g. lactose, glucose, fructose	120 °C, 15 min	Violet fluorescence on a dark blue background.	[31]
Sugars, e.g. lactose, glucose, fructose	120 °C, 15 min	Induced fluorescence; detection limits in nanogram range.	[32]
Glucose, fructose	Infrared lamp or 170 °C each for 3 min	Heating produced stable bluish-white fluorescence ($\lambda_{\text{exc}} = 365 \text{ nm}$ and $\lambda_{\text{fl}} > 400 \text{ nm}$, cut off filter K 400), detection limits; 5–10 ng.	[33]
Sugars, e.g. glucose, rhamnose, xylose etc.	160 °C, 3–4 min or infrared lamp	Induction of brilliant stable fluorescence $\lambda_{\text{exc}} = 365 \text{ nm}$ and $\lambda_{\text{fl}} > 400 \text{ nm}$, (cut off filter K 400), sugar alcohols do not fluoresce; detection limits: 5–10 ng.	[2]
Creatine, creatinine, uric acid in urine and serum	150 °C, 3–4 min	Stable fluorescence $\lambda_{\text{exc}} = 365 \text{ nm}$ and $\lambda_{\text{fl}} > 400 \text{ nm}$, (cut off filter K 400).	[7]
Sugars, e.g. sucrose, ribose, xylose	150 °C, 3–4 min	Induced fluorescence $\lambda_{\text{exc}} = 365 \text{ nm}$ and $\lambda_{\text{fl}} > 400 \text{ nm}$, (cut off filter K 400).	[34]

Procedure Tested

Catecholamines, Serotonin and Metabolites [37]

Layer: NH_2 modified silica gel

Temperature: 150 °C

Reaction

Heteroaromatic ring systems are formed presumably with loss of functional groups, elevated temperatures and probably under the catalytic influence of the aminopropyl groups on the sorbent surface. The compounds so formed are excited to fluoresce by long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Method	Ascending, one-dimensional two-fold development (10 min intermediate drying in stream of cold air) in a trough chamber with chamber saturation.
Layer	HPTLC plates $\text{NH}_2 \text{ F}_{254\text{s}}$ (MERCK).
Mobile phase	Chloroform – 1-propanol – formic acid (50+10+5)
Migration distance	$2 \times 7 \text{ cm}$
Running time	$2 \times 30 \text{ min}$

Detection and result: The chromatogram was dried for 10 min in a stream of warm air and heated to ca. 150 °C under an infrared lamp, on a hot plate or in a drying cupbo for 3 to 4 min.

Noradrenaline ("h R_f " ~15*), adrenaline ("h R_f " ~20), serotonin ("h R_f " ~25), vanilmandelic acid ("h R_f " ~45), creatinine ("h R_f " ~50), hydroxyindoleacetic acid ("h R_f " ~55) and homovanillic acid ("h R_f " ~85) appear on examination in long-wave

*) The figures given here are calculated in the same manner as h R_f , even though two developments were carried out.

length UV light ($\lambda = 365$ nm) as brilliant pale blue fluorescent chromatogram zones on a dark background (Fig. 1A). Vanillic acid ("hR_f" ~90), on the other hand, only fluoresces weakly, but produces appreciable fluorescence quenching on NH₂ layers containing a fluorescence indicator (Fig. 1B).

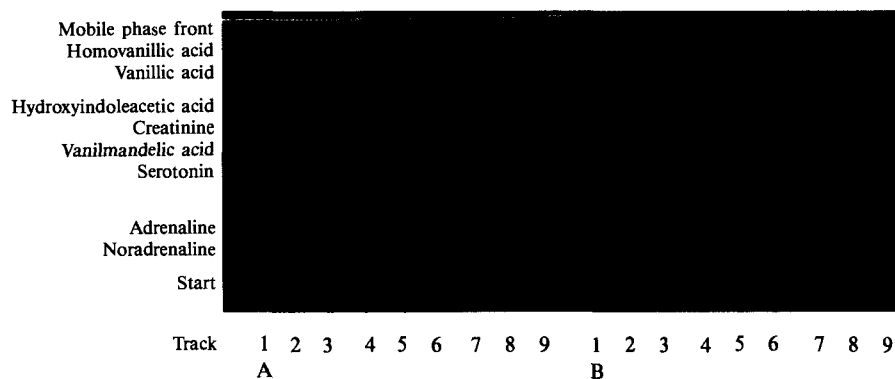


Fig. I: Chromatogram of catecholamines, serotonin and some metabolites together with creatinine: A) examination at $\lambda = 365$ nm, B) examination at $\lambda = 254$ nm.

The visual detection limits for fluorimetric detection are substance-dependent and lie between 5 ng (adrenaline, noradrenaline) and 30 ng (homovanillic acid) substance per chromatogram zone.

In situ quantitation: Fluorimetric evaluation was carried out by excitation at several wavelengths and by measuring the fluorescence emission. (Fig. II).

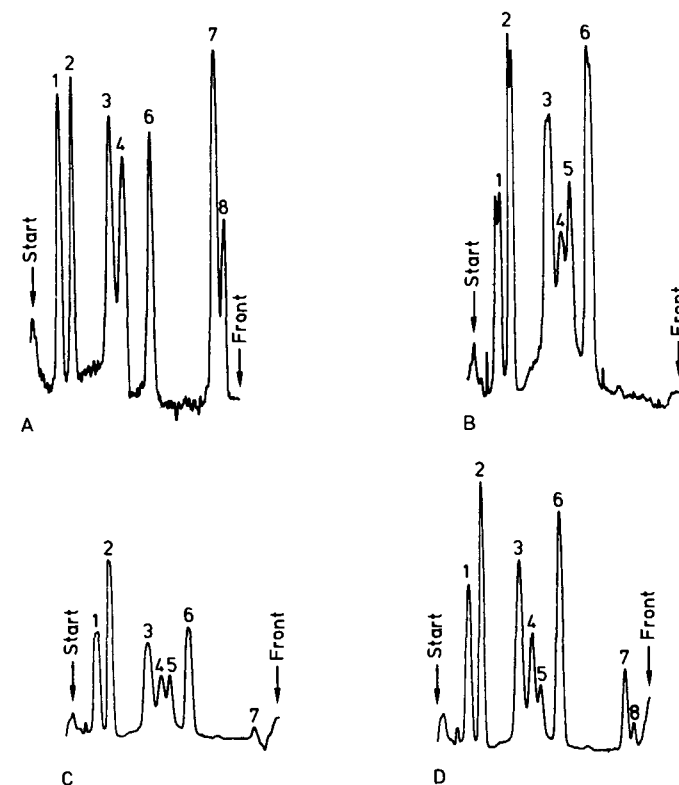


Fig. II: Fluorescence scan of a chromatogram track with 500 ng each of noradrenaline, adrenaline (2), serotonin (3), vanilmandelic acid (4), 5-hydroxyindoleacetic acid (6), homovanillic acid (7) and vanillic acid (8) together with 230 ng creatinine, all per chromatogram zone: measurement at $\lambda_{\text{exc}} = 313$ nm and $\lambda_{\text{fl}} > 390$ nm (cut off filter Fl 39 (A)), $\lambda_{\text{exc}} = 365$ nm and $\lambda_{\text{fl}} > 430$ nm (cut off filter Fl 43 (B)), $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{fl}} > 460$ nm (cut off filter Fl 46 (C)) and $\lambda_{\text{exc}} = 436$ nm and $\lambda_{\text{fl}} > 560$ nm (cut off filter Fl 56 (D)). Under the conditions used for scan C and D some substances appear as double peaks on account of fluorescence quenching at the center of the chromatogram zones as a consequence of the substance concentration being too high.

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1.3 Electrochemical Activation

Electromagnetic radiation energy can be used to stimulate substances to fluoresce after separation by thin-layer chromatography. Its action makes it possible to convert some nonfluorescent substances into fluorescent derivatives. The “active” sorbents often act as catalysts in such processes (cf. Chapter 1.1).

The supply of thermal energy by a heater or IR lamp is a second method of converting the separated substances into fluorescent derivatives. Here too, at about the decomposition temperatures, many substances react to form fluorescent derivatives generally with the catalytic participation of the “active sorbent”. These fluorescent derivatives often provide specific evidence concerning the nature of the substances being detected (cf. Chapter 1.2).

A third possibility of converting nonfluorescent substances to fluorescent derivatives without the use of additional reagents apart from the stationary phase on which separation has been carried out, is to place the plate in a “plasma chamber” for some time.

The application of high tension (e.g. 20 kV, 0.5 MHz) in an evacuated system (0.2 ... 8 torr) causes the residual gas to form a highly ionized mixture of positive and negative ions, electrons, photons and neutral gas molecules. In the presence of “active” sorbents this plasma reacts with the chromatographically separated substances to yield reactive ions and radicals.

Depending on the structures of the substances being investigated the chromatograms are exposed to the effects of the plasma chamber for 5 to 300 s and then heated to 130 °C for 1 to 2 min [1] or irradiated with long-wavelength UV light for 3 min [2]. This causes the formation of fluorescent derivatives with the sorbent again acting as a welcome catalyst.

The residual gas in the plasma chamber can either be a noble gas, nitrogen, methane or hydrogen. Argon yields less intensely fluorescent substances and oxygen permits oxidation reactions that usually lead to nonfluorescent final products. The results obtained by exposing to activated ammonia vapor [1] or acid vapors [3] reveal that displacements can also have positive effects here. Ammonium hydrogen carbonate vapors also behave favorably. SEGURA and GORTO [4] used them for the induction of fluorescence using thermal activation many years ago.

Some examples of substances present in chromatogram zones being made highly fluorescent by exposure to electrochemical stimulation in an atmosphere of nitrogen are listed in Table 3 [1]. The plates used were “Permakote” silica gel layers containing an organic binder. However, silica gel 60 HPTLC plates (MERCK) were also employed; these yield a considerable background fluorescence on account of the organic binder they contain.

It is also possible to ionize the gases at normal atmospheric pressure instead of using a plasma chamber [2]. In this case the gas is passed through a high tension field at a flow rate of 5 to 30 L/min (spark discharge: 20 kV, 0.5 MHz) and blown onto the chromatogram (Fig. 20). As in the case of the electric vacuum discharge-chamber described above the chromatographed substances are activated to intense fluorescence emission. Under favorable conditions (low background layer fluorescence) it is still possible to detect 1 ng chlorpromazine or $n\text{-C}_{22}\text{H}_{46}$ visually. Hence this mode of activation is just as sensitive as the thermochemically generated fluorescence described in Chapter 1.2 [3].

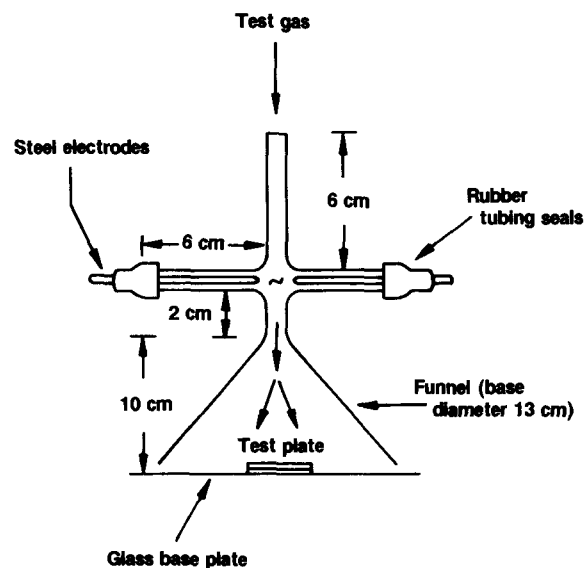


Fig. 20: Schematic representation of an electric spark discharge chamber for the activation of gases at normal atmospheric pressure for the production of fluorescence in substances separated by thin-layer chromatography [2].

Table 3: Some substances that produce intense fluorescence when treated with ionized nitrogen after they have been chromatographed [1].

Substance	Exposure time [s]	Substance	Exposure time [s]
Cholesterol	60	Oleic acid	180
Cholesteryl pelargonate	60	Morphine	180
Progesterone	60	Codeine	180
Testosterone	60	Cocaine	180
Dieldrin	60	Dimerol	180
Tetrahydrocannabinol	60	Phenobarbital	180
Inositol	60	Chlorpromazine	180
Lauryl alcohol	180	d-Amphetamine sulfate	180
$n\text{-C}_{22}\text{H}_{46}$	180	Methadone	180
Phenol	180		

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2 Reagents for the Recognition of Functional Groups

The aim of most screening methods is to produce a yes/no decision, concerning whether the concentration of a certain substance in a sample exceeds a given limit concentration or not. For instance, if the concentration of a substance lies below a permitted maximum concentration then there is probably no need to analyse the sample. However, if the content is in the region of or above the permitted limit, then the result must be confirmed by means of an exact quantitative determination.

Such yes/no decisions are of great importance in foodstuffs control and environmental analysis. They also play an important role in pharmacy in the form of content uniformity tests. Without suitable screening methods for rapid detection of positive samples it would scarcely be possible to carry out economic doping controls and toxicological investigations or to recognize medicament abuse.

Thin-layer chromatography is an excellent screening method because:

- Many samples can be chromatographed alongside each other.
- It is possible to chromatograph reference substances on the same TLC/HPTLC plate and thus compare unknown samples in the same defined system.
- Only a few milliliters of mobile phase are required for the separations so that there are scarcely any disposal problems.
- The whole chromatogram can be taken in at a glance and an immediate comparative evaluation can be made.
- It is often possible to carry out any clean-up step that may be necessary in the concentrating zone of a suitable chromatographic plate, in any event clean-up is less complex than for other forms of chromatography.
- There is no necessity to regenerate the sorbent since TLC/HPTLC plates are generally used twice.

The unequivocal recognition or exclusion of particular substances in question is of especial importance for such screening methods. As far as the chemist is concerned it can involve a deliberate search for substances with particular functional groups; particular questions that might require answering might include the following:

- Does the sample contain a substance with a carbonyl group or one that has been produced by oxidation of an alcoholic OH group?
- Does the reduction of the sample molecule lead to the formation of substances containing amino groups?

- Are there substances in the sample capable of coupling reactions and where are they to be found in the chromatogram?

These few questions serve to demonstrate that there must be great interest in characterizing chemical compounds by means of their reactive functional groups. The most important group-specific reagents for postchromatographic derivatization are listed alphabetically in Table 4 below:

Table 4: Reagents suitable for the recognition of functional groups.

Functional group	Reagent	Remarks	Reference
Acetylene compounds	Dicobaltoctacarbonyl	Formation of colored complexes. After the reagent excess has been washed out, reaction with bromine vapor yields cobalt bromide, which reacts with α -nitroso- β -naphthol to yield red chromatogram zones on an almost colorless background.	[11]
Aldehydes	4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald reagent)	Aldehydes yield violet chromatogram zones on a whitish-yellow background. Some alcohols form yellow to orange-colored chromatogram zones.	[2]
Aldehydes	2,4-Dinitrophenyl-hydrazine	Formation of colored hydrazones or osazones. It is possible to distinguish between saturated and unsaturated hydrazones using potassium hexacyanoferrate(III) [5].	[3, 4]
Aldehydes	Hydrazine sulfate + hydrochloric acid	Aromatic aldehydes yield colored hydrazones.	[6]
Alcohols	4-(4-Nitrobenzyl)-pyridine	Amino compounds, esters and ethers do not interfere, but phenols and acids as well as epoxides, olefins and substances containing labile halogen probably do.	[7]
Alcohols (diols, polyols, sugars)	Lead(IV) acetate — dichlorofluorescein	Diol cleavage of vicinal diols, e.g. sugars, sugar alcohols. The lead tetraacetate consumed is no longer available to decompose the fluorescent dichlorofluorescein.	[3, 8]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Amines (primary)	Ninhydrin	Reddish or bluish chromatogram zones are produced, amino sugars and amino acids also react. Unexpectedly ascorbic acid also reacts.	[3, 9]
Amines (primary aliphatic and aromatic)	Diphenylboric anhydride + salicylaldehyde (DOOB)	Fluorescent reaction products are produced.	[3, 10]
Amines (primary)	<i>o</i> -Phthalaldehyde (OPA)	In the presence of mercaptoethanol <i>o</i> -phthalaldehyde reacts with primary amines and amino acids to yield fluorescent isoindole derivatives.	[3, 11]
Amines (primary)	Trinitrobenzenesulfonic acid (TNBS)	On heating primary amines react with TNBA to yield intensely colored MEISENHEIMER complexes. Amino acids also react.	[3, 12]
Amines (primary)	Fluorescamine	Primary aliphatic and aromatic amines yield fluorescent derivatives. Primary aromatic amines yield stable yellow-colored derivatives that can be eluted from the TLC layer [16].	[3, 13, 15]
Amines (primary aromatic)	Sodium nitrite + α -naphthol or BRATTON-MARSHALL reagent	Diazotization of the primary amine followed by coupling with α -naphthol or N-(1-naphthyl)-ethylenediamine. Sulfonamides also react [18].	[3, 14]
Amines (primary aromatic)	4-(Dimethylamino)-benzaldehyde + acid	Alkaloids and indole derivatives also react [19].	[17]
Amines (capable of coupling)	Fast blue salt B, fast blue salt BB, fast black salt K, diazotized sulfanilic acid (PAULY's reagent), diazotized sulfanilamide or 4-nitroaniline	Intensely colored azo dyes are produced. Catecholamines [20], imidazoles [21] and phenols also react.	[3, 15]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Amines (primary and secondary)	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	Fluorescent 4-nitrobenzofurazan derivatives are produced. Phenols and thiols also react.	[3]
Amines (primary and secondary aromatic)	<i>p</i> -Chloranil	The reaction depends on the catalytic effect of silica gel. Monochlorobenzene, as solvent for the reagent, also contributes. There is no reaction on cellulose layers.	[17, 22]
Amines (secondary aliphatic and alicyclic)	Sodium nitroprusside + acetaldehyde	Secondary aliphatic and alicyclic amines yield blue-colored chromatogram zones (e.g. morpholine, diethanolamine).	[23, 24]
Amines (long-chain primary, secondary and tertiary plus quaternary ammonium salts)	Cobalt(II) thiocyanate	Long-chain primary, secondary and tertiary amines and long-chain quaternary ammonium salts yield blue chromatogram zones on a pink background.	[25]
Carboxyl groups (carboxylic acids)	Indicators, e.g. bromocresol green, bromocresol green + bromophenol blue + potassium permanganate, bromocresol purple, methyl red + bromothymol blue	Detection depends on the color change of the indicator in acid medium. Quaternary ammonium salts give a color change in some cases [2].	[2, 26] [3, 27]
Carboxyl groups (carboxylic acids)	2,6-Dichlorophenol-indophenol (TILLMANN's reagent)	Organic acids release the red undissociated acid from the blue mesomerically stabilized phenolate anion. Reductones reduce the reagent to a colorless compound.	[3] [28, 29]
Carboxyl groups (carboxylic acids)	Aniline + aldose (e.g. glucose)	The action of acid causes glucose to be converted to furfural which reacts with aniline to yield a colored product.	[3]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Halogen derivatives	Silver nitrate, ammoniacal (DEDONDER's, TOLLENS' or ZAFFARONI's reagent)	Halogen compounds yield black chromatogram zones on a pale gray background.	[2]
Ketones	2,4-Dinitrophenylhydrazine	Formation of colored hydrazones or osazones. It is possible to distinguish between saturated and unsaturated hydrazones using potassium hexacyanoferrate(III) [5].	[3, 4]
Nitro derivatives	Benzylcyanide + benzyltrimethylammonium hydroxide	Nitro compounds, e.g. explosives, or pesticides containing nitro groups yield gray to bluish-green chromatogram zones on a brownish background.	[30]
Peroxides	1-Naphthol + N ⁴ -ethyl-N ⁴ -(2-methanesulfonamidoethyl)-2-methyl-1,4-phenylenediamine (peroxide reagent)	A quinonimine dyestuff is produced on reaction with peroxides.	[3]
Peroxides	Iron(II) sulfate + ammonium thiocyanate	Peroxides rapidly oxidize iron(II) to iron(III) ions which react to yield brown-red iron(III) thiocyanate complexes.	[31, 32]
Peroxides	Potassium iodide + starch	Peroxides release free iodine which forms a blue complex with the starch.	[17, 33]
Peroxides	N,N-Dimethyl-1,4-phenylenediamine (N,N-DPDD), N,N,N',N'-tetramethyl-1,4-phenylenediamine (TPDD)	Peroxides, e.g. alkyl hydroperoxides, oxidize N,N-DPDD to WURSTER's red and TPDD to WURSTER's blue.	[17] [34]
Phenols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	Fluorescent 4-nitrobenzofuran derivatives are produced. Primary and secondary aromatic amines and thiols also react.	[3]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Phenols (capable of coupling)	Fast blue salt B, fast blue salt BB, fast black salt K, diazotized sulfanilic acid (PAULY's reagent) diazotized sulfanilamide or 4-nitroaniline	Intensely colored azo dyes are formed. Catecholamines [20, 35], imidazoles [21] and amines capable of coupling also react.	[3, 17]
Thiols, thioethers, disulfides	Sodium metaperiodate + benzidine	Substances with divalent sulfur yield white chromatogram zones on a blue background.	[36]
Thiols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	Fluorescent 4-nitrobenzofuran derivatives are formed. Primary and secondary aromatic amines and phenols also react.	[3]

It should be remembered that the group-specific reagents listed in Table 4 are rarely completely selective, for there are almost always a few substances that do not contain the particular group and yet give a comparable reaction! The detection of ascorbic acid with ninhydrin is a spectacular example of this. Ninhydrin usually reacts with primary amino groups. The other side of the coin is that there is scarcely a more sensitive reagent for vitamin C even though this substance does not contain an amino group.

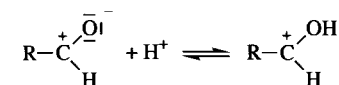
In addition the role played by the sorbent on which the chromatography is carried out must not be neglected. For instance, it is only on aluminium oxide layers and not on silica gel that it is possible to detect caffeine and codeine by exposure to chlorine gas and treatment with potassium iodide – benzidine [37]. The detection limits can also depend on the sorbent used. The detection limit is also a function of the hR_f value. The concentration of substance per chromatogram zone is greater when the migration distance is short than it is for components with high hR_f values. Hence, compounds with low hR_f values are more sensitively detected.

These reactions at particular functional groups of the sample molecule are closely related in an inverse sense with those reagents which bring their own functional group into the molecule. The numerous "aldehyde – acid" reactions are an example. Numerous monographs of such reactions are already included in Volume Ia. Their reac-

tivity depends on the ready polarizability of the carbonyl group as a result of the inductive effect of the carbonyl oxygen.



The carbonyl group also possesses electrophilic properties at the carbon atom and nucleophilic properties at the oxygen atom. Nucleophilic attack of the carbonyl group is favored if this is attached to an aromatic ring (inductive effect) and there is also a methoxy or phenolic OH group present in the 4-position. Changing a neutral reaction medium by proton addition has the same effect.



Three large groups of substances are suitable nucleophilic reaction partners, namely:

- bases
- C-H acidic compounds
- crypto bases.

The boundaries between these groups are not always easily delineated. Nevertheless the classification is useful in practice [38]. A selection of "base" reactions is listed in Table 5.

Table 5: Reactions of bases with carbonyl compounds, a selection.

Reactants	Reaction/reaction product
$\text{>C=O} + \text{H}-\text{O}^--\text{H} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C} \\ \\ \text{OH} \end{array}$	Hydrates
$\text{>C=O} + \text{H}-\text{O}^--\text{R} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C} \\ \\ \text{OR} \end{array} \xrightarrow[\text{-H}_2\text{O}]{+\text{ROH (H}^+)} \begin{array}{c} \text{OR} \\ \\ \text{>C} \\ \\ \text{OR} \end{array}$	Acetals and ketals
$\text{>C=O} + \text{H}-\text{S}-\text{R} \rightleftharpoons \begin{array}{c} \text{SR} \\ \\ \text{>C} \\ \\ \text{SR} \end{array}$	Mercaptals

Table 5: (continued)

Reactants	Reaction/reaction product
$\text{>C=O} + \begin{array}{c} \text{H} \\ \\ \text{N}-\text{R} \\ \\ \text{H} \end{array} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C} \\ \\ \text{NHR} \end{array} \xrightarrow{-\text{H}_2\text{O}} \text{>C=NR}$	SCHIFF's bases
$+ \text{NH}_2-\text{OH} \longrightarrow \text{>C=NOH}$	Oximes
$+ \text{NH}_2-\text{NH}-\text{R} \longrightarrow \text{>C=N-NHR}$	(Substituted) hydrazones
$+ \text{NH}_2-\text{NH}-\text{CO}-\text{NH}_2 \longrightarrow \text{>C=N-NH-CO-NH}_2$	Semicarbazones
<hr/>	
$\begin{array}{c} \text{CH} \\ \\ \text{>C=O} \end{array} + \text{H-NR}_2 \rightleftharpoons \begin{array}{c} \text{CH} \\ \\ \text{>C} \\ \quad \\ \text{OH} \quad \text{NR}_2 \end{array} \xrightarrow{-\text{H}_2\text{O}} \begin{array}{c} \text{CH} \\ \\ \text{C}=\text{C} \\ \quad \\ \quad \text{NR}_2 \end{array}$	Enamines
<hr/>	
$\text{>C=O} + \begin{array}{c} \text{OH} \\ \\ \text{S}=\text{O} \\ \\ \text{ONa} \end{array} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C} \\ \quad \\ \text{S}=\text{O} \\ \\ \text{ONa} \end{array}$	Bisulfite addition compounds
<hr/>	
$\text{>C=O} + \begin{array}{c} \text{H} \\ \\ \text{C}=\text{P}(\text{C}_6\text{H}_5)_3 \\ \\ \text{R} \end{array} \xrightarrow{-(\text{C}_6\text{H}_5)_3\text{PO}} \begin{array}{c} \text{H} \\ \\ \text{C}=\text{C} \\ \\ \text{R} \end{array}$	WITTIG reaction
<hr/>	
$\text{>C=O} + 2\text{e}^- + 2\text{H}^+ \longrightarrow \text{>CH-OH}$	Hydrogenation to alcohols

C-H acidic compounds do not possess any basic properties. But they can form anions in the presence of strong bases, and these possess sufficiently strong nucleophilic properties to be able to add to a polarized carbonyl group. Examples are listed in Table 6.

Table 6: Reaction of carbonyl compounds (aldehydes, ketones) with C-H acidic compounds: a selection.

Reactant	Reaction/reaction product
$\text{>C=O} + \text{H}-\text{C}\equiv\text{N} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C}-\text{C}\equiv\text{N} \end{array}$	Cyanhydrins
$\text{>C=O} + \text{H}-\text{C}\equiv\text{C}-\text{H} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C}-\text{C}\equiv\text{C}-\text{H} \end{array}$	Ethynylation
$\text{>C=O} + \begin{array}{c} \text{O} \\ \\ -\text{CH}_2-\text{C} \\ \\ \text{H(R)} \end{array} \rightleftharpoons \begin{array}{c} \text{OH} \\ \\ \text{>C}-\text{CH}-\text{C} \\ \quad \\ \quad \text{H(R)} \end{array} \xrightarrow{-\text{H}_2\text{O}} \begin{array}{c} \text{O} \\ \\ \text{>C}=\text{C}-\text{C} \\ \quad \\ \quad \text{H(R)} \end{array}$	Aldol addition
	Aldol condensation
$\text{Ar}-\text{C}=\text{O} + \begin{array}{c} \text{CH}_3-\text{C}=\text{O} \\ \\ \text{CH}_3-\text{C}=\text{O} \end{array} \xrightarrow{-\text{CH}_3\text{COOH}} \text{Ar}-\text{CH}=\text{CH}-\text{C}=\text{O}$	PERKIN reaction
$\text{Ar}-\text{C}=\text{O} + \begin{array}{c} \text{CH}_2-\text{C}=\text{O} \\ \\ \text{NH}-\text{C}=\text{O} \\ \\ \text{C}_6\text{H}_5 \end{array} \xrightarrow{-2\text{H}_2\text{O}} \begin{array}{c} \text{Ar}-\text{CH}=\text{C}-\text{C}=\text{O} \\ \quad \\ \text{N} \quad \text{O} \\ \quad \\ \text{C}_6\text{H}_5 \end{array}$	ERLENMEYER reaction
$\text{>C=O} + \begin{array}{c} \text{CH}_2-\text{COOR} \\ \\ \text{Cl} \end{array} \xrightarrow{-\text{HCl}} \begin{array}{c} \text{O} \\ \diagup \quad \diagdown \\ \text{C}-\text{CH}-\text{COOR} \end{array}$	DARZEN's glycidic ester synth
$\text{>C=O} + \begin{array}{c} \text{X} \\ \\ \text{CH}_2 \\ \\ \text{Y} \end{array} \xrightarrow{-\text{H}_2\text{O}} \begin{array}{c} \text{X} \\ \\ \text{>C}=\text{C} \\ \\ \text{Y} \end{array}$	KNOEVENAGEL condensation

It is not possible to draw a sharp boundary between the reaction of C-H acidic substances (e.g. aldol reactions) and the reactions of *cryptobases*. The cryptobases include organometallic compounds and metal hydrides, whose alkyl residues or hydride atoms are rendered negative by the +I effect of the metal and, hence, are readily transferred to a carbonyl group together with their bonding electrons. Hydrogen atoms attached to carbon atoms can also react when they are subject to great electron pressure. Here the presence of LEWIS acids induces reactions which usually take place via a cyclic transition state where all electron transfers are simultaneous. The named reactions listed in Table 7 are examples.

Table 7: Reactions of carbonyl groups with cryptobases.

Reactant	Reaction/reaction product
$\text{>C=O} + \begin{array}{c} \text{R} \\ \\ \text{CH-OH} \\ \\ \text{R} \end{array} \xrightleftharpoons{\text{Al(OR)}_3} \begin{array}{c} \text{CH-OH} \\ \\ \text{R} \end{array} + \begin{array}{c} \text{R} \\ \\ \text{C=O} \\ \\ \text{R} \end{array}$	MEERWEIN-PONNDORF reduction OPPENAUER oxidation
$\text{R-CHO} + \text{R-CHO} \xrightarrow{\text{Al(OR)}_3} \text{R-COOCH}_2\text{-R}$	CLAISEN-TISCENKO reaction
$\text{R-CHO} + \text{R-CHO} + \text{H}_2\text{O} \xrightarrow{\text{OH}^-} \text{R-COOH} + \text{R-CH}_2\text{OH}$	CANNIZZARO reaction
$\text{>C=O} + \text{HN} \begin{array}{c} \diagup \\ \diagdown \end{array} + \text{HCOOH} \longrightarrow \begin{array}{c} \text{CH-N} \\ \diagup \quad \diagdown \end{array} + \text{CO}_2 + \text{H}_2\text{O}$	LEUCKART-WALLACH reaction
$\text{>C=O} + \text{R-MgX} \longrightarrow \begin{array}{c} \text{OMgX} \\ \\ \text{C} \\ \\ \text{R} \end{array} \xrightarrow[\text{-XMgOH}]{+\text{H}_2\text{O}} \begin{array}{c} \text{OH} \\ \\ \text{C} \\ \\ \text{R} \end{array}$	GRIGNARD reaction
$4 \text{>C=O} + \text{LiAlH}_4 \longrightarrow (\text{CH-O})_4 \text{AlLi} \xrightarrow{\text{H}_2\text{O}} 4 \text{>CH-OH}$	Reduction with complex hydrides

These basic reaction mechanisms occur with many of the reagents treated in Volumes 1a and 1b. The following examples can be listed:

- 4-Aminobenzoic acid + sugars
- 2-Aminodiphenyl – sulfuric acid + carbonyl compounds
- 4-Aminohippuric acid + monosaccharides
- 4-Aminohippuric acid – phthalic acid + sugars
- Aniline – diphenylamine – phosphoric acid + sugars
- Aniline – phosphoric acid + carbohydrates
- Aniline – phthalic acid + sugars
- Anisaldehyde – phthalic acid + sugars
- *p*-Anisidine – phthalic acid + oligosaccharides
- 4-(Dimethylamino)-cinnamaldehyde – hydrochloric acid + indole derivatives
- 2,4-Dinitrophenylhydrazine + carbonyl compounds
- EHRlich's REAGENT + indole derivatives
- EP reagent + terpenes
- MARQUIS' reagent + alkaloids
- 1,2-Phenylenediamine – trichloroacetic acid + ascorbic acid
- PROCHAZKA's reagent + indole derivatives
- VAN URK's reagent + indole derivatives

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3 Reagent Sequences

Thin-layer chromatography has the great advantage that the result of the separation is stored – usually invisibly – on the TLC/HPTLC plate as on a diskette. In such cases it needs developing or detecting, rather like an exposed film. This can now be done on-line or off-line so that the analyst can decide which method to use to detect the separated substances.

Physical methods, some of which can be applied in the on-line mode (Fig. 8) and *physiological methods* providing information concerning the effectivity of the separated substance can be used. A later volume will treat these bioautographic methods which can be subdivided into bioassays (off-line) and bioautogrammes (on-line).

The *microchemical detection* methods are the most economical methods and the simplest for the chemist to carry out. No expensive apparatus is required and in certain circumstances they can be combined with the two detection methods mentioned above.

The aim of microchemical investigations on TLC/HPTLC plates is to provide information concerning identity and/or purity. Quantitation is not usually carried out but is possible at any time. The simple absence of a reaction is frequently taken as evidence of the absence of a particular substance.

If color reactions occur these serve to help characterize the substance. They can only ever act as a pointer to the presence of a substance, but never as proof even when accompanied by a separation process. Unequivocal identification requires a mosaic of many pieces of information (hR_f values, color reactions UV/VIS, IR, Raman, mass spectra etc).

As is well known the difficulty of analysis of a sample increases as its complexity increases. Analysis usually commences with a rather nonspecific clean-up step and requires that the separation step that follows be highly selective and depends on a detection step that is as specific as possible. As the selectivity of detection increases there is also an increase in the reliability of the identification and it is possible to reduce the demands made on the selectivity of the preceding separation method. This is the case for radiometric and enzymatic methods and also explains the popularity of fluorescence measurements. The latter obtain their selectivity from the freedom to choose excitation and measurement wavelengths.

Color reactions are more or less clearly defined reactions of the substances with suitable reagents. Substance-specific reactions are not usually available, e.g. many compounds with aromatic skeletons give both a positive VITALI-MORIN reaction [1–4] and a positive MARQUIS reaction [4]. Again, numerous aldehydes react with electron-rich compounds in acidic medium to yield colored substances (cf. Chapters 2 and 3.1).

Reactions can be exploited more specifically if it is known that particular functional groups are present [cf. Chapter 2]. They still do not allow direct identification, but they increase the specificity of the evidence. The chromatographic separation carried out before detection also contributes to this. This reduces the number of potential components. However, this does not exclude the possibility that there might be several substances in the particular part of the chromatogram involved. This not only applies to thin-layer chromatography but also applies with equal force to other microanalytical separation methods (GC, HPLC).

It is often possible to increase the selectivity of detection by carrying out a sequence of reactions on one and the same chromatographic plate – a technique that is only possible in thin-layer chromatography. In principle it is possible to distinguish between two sorts of reagent sequence (“TYPE A” and “TYPE B”), which are discussed in this and the next volume.

The examples that are treated below are those sequences where all steps – except the last – are preparations for a color or fluorescence derivatization reaction which is carried out in the last step, i.e. they can be regarded as a sort of selective in situ pretreatment for a final detection reaction. Such reaction sequences are frequently necessary because all the reagents cannot be mixed together in a single solvent, or because it is necessary to dry, heat or irradiate with UV light between the individual reaction steps. The detection of aromatics by the reaction sequence “nitration – reduction – diazotization – couple to form an azo dye” is an example of this type (Fig 21).

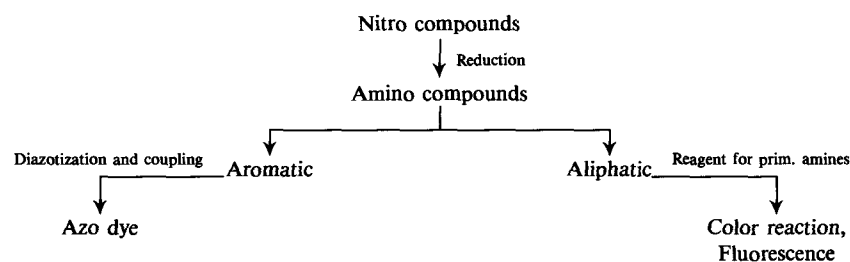


Fig. 21: Reaction scheme for the detection of aromatics, by means of the reaction sequence, nitration, reduction, diazotization and coupling to an azo dye, and of aliphatic nitro compounds by detection of the primary amino group produced on reduction.

The “true” reaction sequences that will be described in Volume 1c are frequently used in toxicological analysis, since the unequivocal identification of medicaments, intoxicants and addictive drugs in body fluids almost always requires the simultaneous detection of many possible substances with completely different chemical characteristics. For

this purpose various staining reagents, each of which can be used individually for a particular substance class, are applied consecutively to the same chromatogram. After each application the result is evaluated under visible or UV light and the result recorded photographically, if necessary, for it is possible that the result obtained might be destroyed or made unrecognizable by the next reaction step. (Fig. 22).

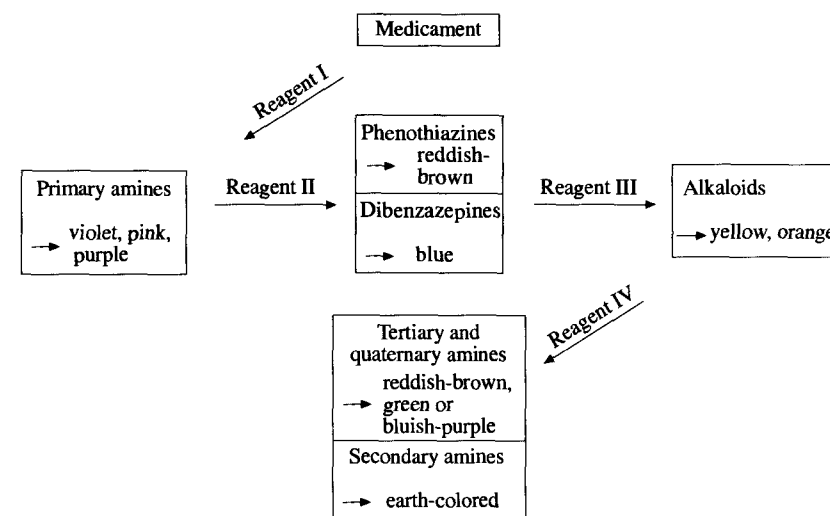


Fig. 22: Scheme for type B reagent sequence (cf. the reagent sequence “ninhydrin – iron(III) chloride – DRAGENDORFF – potassium iodoplatinate” [6]).

Therefore, such sequential in situ reactions are always carried out either in order to prepare a substance for a color reaction that is to follow or to increase the amount of information that is obtained by exploiting a combination of different independent reactions. This provides information that could not be obtained using one single reagent.

Both types of reagent sequence are frequently used when the samples are complex in nature (plant extracts, urine, environmental samples etc.). It can happen that an excess of reagent A could interfere with the reaction of the following reagent B. With the dipping technique it is also possible that reagent transfer might occur making it only possible to use reagent B once.

In such cases it is possible to use intermediate “rinsing troughs” in the form of appropriately prepared dipping chambers (Fig. 23) or diffusion destaining apparatus, such as is used in electrophoresis (Fig. 24). These can also be used sometimes to destain the layer background when single reagents are used (cf. potassium hexaiodoplatinate reagent).

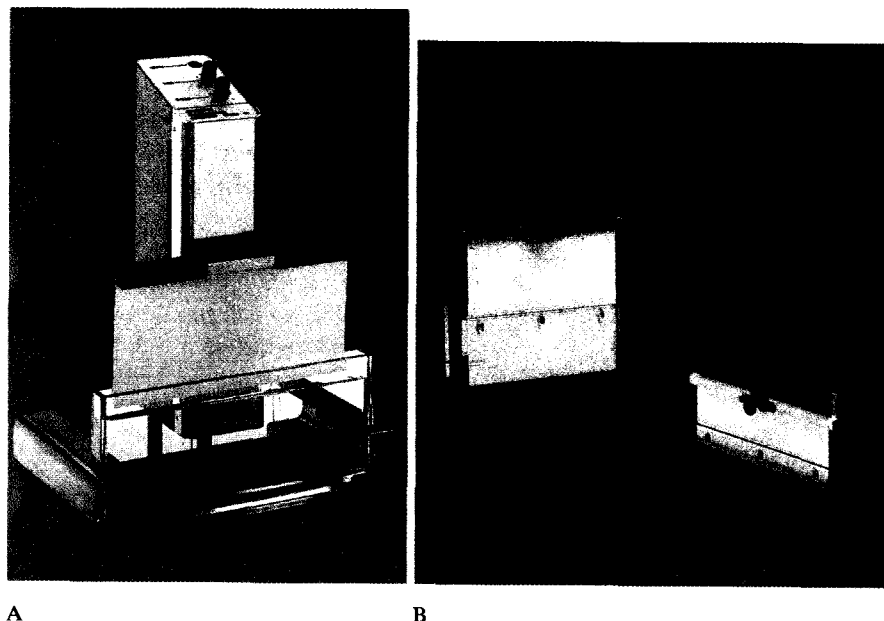


Fig. 23: Chromatogram dipping device III (CAMAG) (A), (DESAGA) (B).

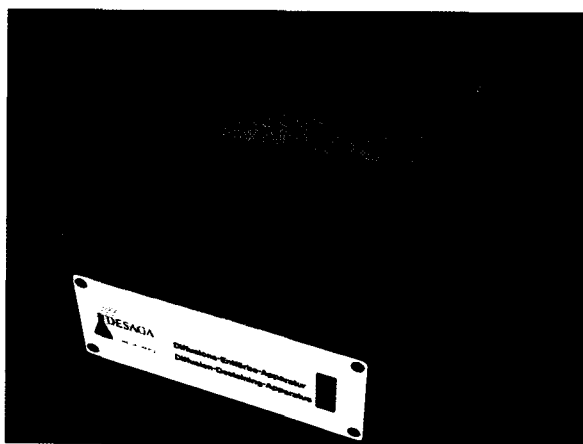


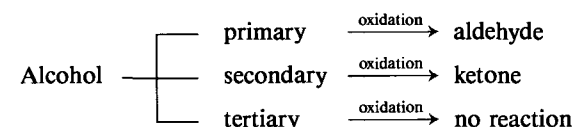
Fig. 24: Diffusion destaining apparatus (DESAGA). The dish contains the wash liquid and is periodically tilted so that the reagent excess is removed from the chromatogram plate.

Thin-layer chromatography usually involves the adsorption chromatographic separation of substance mixtures into polarity groups. It is well known that clean looking chromatographic peaks can "hide" several substances. For instance, primary, secondary and tertiary alcohols are to be found at very nearly the same R_f .

It is only possible to distinguish them on the basis of prechromatographic reaction. Here it is possible to acetylate primary alcohols without difficulty while leaving tertiary alcohols unreacted under the conditions chosen.

Secondly it is possible to carry out "functional chromatography" within the framework of a two-dimensional development [6-8]. The first separation is followed by an in situ reaction of the sample substance on the layer; the chromatogram is then developed perpendicular to the direction of the first chromatogram (SRS technique). The decision concerning the type of alcohol, is then made on the basis of the position of the chromatogram zones: esters migrate appreciable further than their parent alcohols and acids.

Thirdly, if it is not possible to apply the SRS technique, it can be established whether a primary, secondary or tertiary alcohol is present by oxidizing the alcohol on the chromatographic zone and then subjecting the oxidation product to a detection reaction. On oxidation primary alcohols form aldehydes, secondary alcohols ketones and tertiary alcohols are not oxidized.



If a group-specific reagent is now used, e.g. one that is chosen to react specifically with the reducing properties of aldehydes (ammoniacal silver nitrate solution) or react with ketones (2,4-dinitrophenylhydrazine [9]) it is very simple to determine which form of alcohol is present in the sample.

This example demonstrates the following:

- The derivatization is always carried out with an aim in mind,
- Group-specific reagents can provide evidence to characterize the substance,
- The desired results can be obtained by multiple chromatography or multiple in situ reaction at the same chromatogram zone.

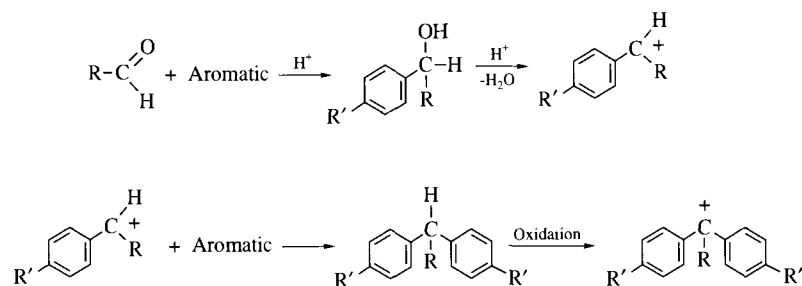
This leads to savings of time and materials.

The specific detection of aromatic nitro compounds is a second example. These can be converted by reduction to primary amines, which are then diazotized and coupled to yield azo dyes (cf. reagent sequence “Titanium(III) chloride – BRATTON-MARSHALL reagent”). Sodium nitrite–naphthol reagent, diazotized sulfanilic acid and other reagents specific for amino groups (e.g. ninhydrin, fluorescamine, DOOB, NBD chloride [9]) can also be used in the second stage of the reaction (Fig. 21).

Finally some reagent sequences are included that lead to a selectivity increase on detection. These frequently yield cationic, anionic or neutral polymethynes, azo dyes, quinonoid or indigoid dyes [4]. Polymethyne radicals, polyenes and metal and charge transfer complexes are also represented [4]. With such a large number of possibilities it is self-evident that any list will be incomplete and that some reaction sequences can be assigned to various reaction categories. The examples included are taken from the literature and have been included without their having been checked by the authors. A gray bar at the side of the text makes this evident.

3.1 Electrophilic Substitutions

Many known color reactions involve electrophilic substitution at an electron-rich aromatic or heteroaromatic (cf. “4-(dimethylamino)-benzaldehyde – acid reagents” and “vanillin reagents”). Here aliphatic or aromatic aldehydes react in acid medium to yield polymethyne cations which are intensely colored di- or triarylcarbenium ions [4, 10].



Formaldehyde (MARQUIS [11] and PROCHAZKA reagent), furfural, 4-methoxybenzaldehyde, 4-(dimethylamino)-cinnamaldehyde and 4-(dimethylamino)-benzaldehyde (EHR- LICH's, VAN URK's, MORGAN-ELSON or EP reagents) react according to this scheme with

phenols, pyrrole or indole derivatives as reaction partners [4]. Examples are to be found in the reagent monographs.

Primary alcohols can be selectively detected using reagent sequences involving an initial oxidation to yield aldehydes that are then reacted in acid medium with electron-rich aromatics or heteroaromatics, according to the above scheme, to yield intensely colored triphenylmethane dyes.

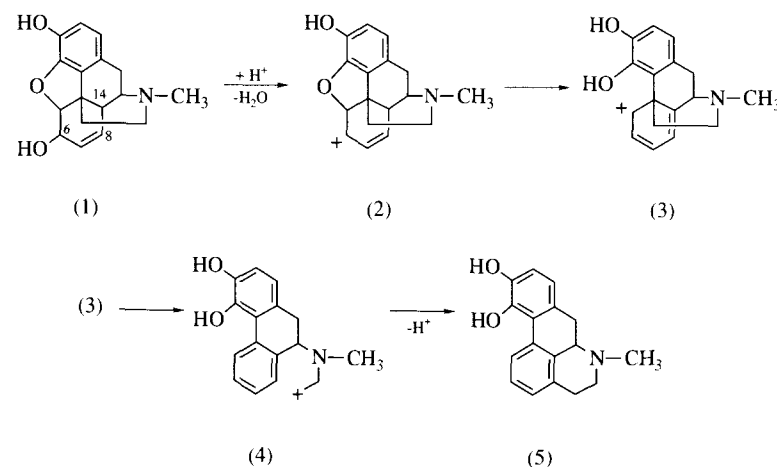
Secondary alcohols yield ketones on oxidation and these can be reacted with 2, dinitrophenylhydrazine to yield the corresponding colored hydrazones.

These examples form a link with derivatizations depending on redox reactions.

3.2 Oxidations and Reductions

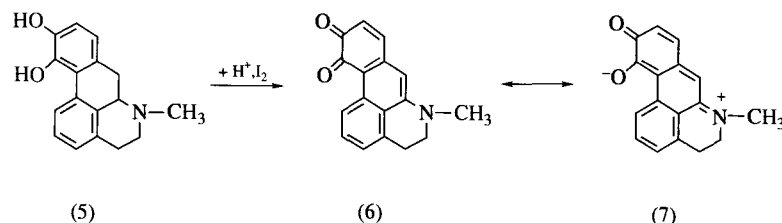
Substances that can oxidize to form a system of conjugated double bonds are frequently oxidized by atmospheric oxygen, iodine or iron(III) salts. The products are chromophoric systems frequently containing *ortho*- or *para*-quinoid structure.

For instance morphine (1) can be detected by the formation of various quinones via apomorphine as intermediate [4, 12, 13]. All morphines with an OH group in the position and a $\Delta^{7,8}$ double bond (codeine, ethylmorphine etc.) first undergo an acid catalyzed rearrangement according to the following scheme [12]:

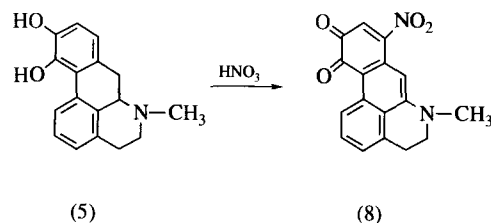


Here the alcoholic hydroxyl is first protonated and then eliminated as water. The allylcarbenium ion (2) is initially stabilized by elimination of the proton at C-14. Then the ether link is opened after protonation of the ring oxygen with the formation of carbenium ion (3), whereby the neighboring C-C bond of the piperidine ring is cleaved with aromatization of the C ring. The carbenium ion (4) formed is stabilized by elimination of a proton and ring closure to apomorphine (5).

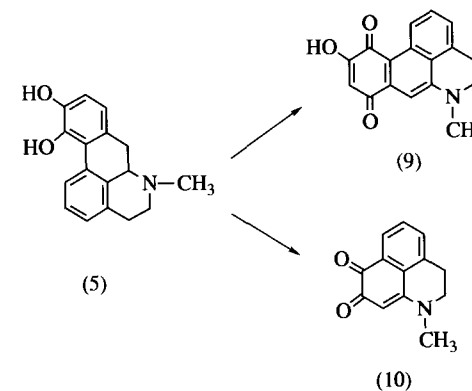
The *ortho* diphenolic structure of apomorphine makes it a strongly reducing substance; hence, in acid medium it forms the blue colored *ortho*-quinone (6) with iodine or other oxidizing agent which is in equilibrium with its zwitterionic limiting structure (7) (PELLARGI's reaction [14]).



The HUSEMANN and ERDMANN identification reactions for morphine and codeine in the DAB 9 (German Pharmacopoeia) involve the formation of the red-colored *ortho*-quinone (8) via apomorphine (5) under the influence of nitric acid with the simultaneous nitration of the benzene ring [15].

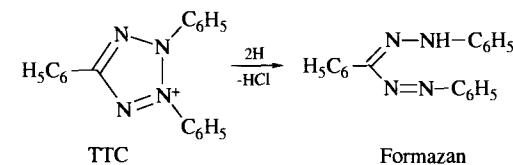


In alkaline medium, in contrast, apomorphine (5) yields the two quinones (9) and (10) [16]:



Redox reactions can naturally lead to the formation of numerous other colored substances in addition to quinonoid and radical chromophores. These include:

- The thalleiochin reaction for the specific detection of quinine alkaloids carrying an oxygen group at C-6 of the quinoline nucleus (e. g. quinine and quinidine) [17], or
- KOBER reaction of phenolic steroids with strong acids leading to polymethine compounds [18, 19].
- The well-known triphenyltetrazolium chloride (TTC) reaction for the detection of α -ketosteroids, pyridinium carbinols and pyridinium glycols can also be included here [20–23]. The chromophore system of the red-colored formazan dye product by reduction of the TTC is composed of highly conjugated double bonds resulting from the combination of a phenylhydrazone group with an azo group:



The following examples of reagent sequences, which include the reagent “/ monium monovanadate – *p*-anisidine” described in the second part of the book, can also be classified as redox reactions.

4-Aminobenzenesulfonic Acid/ 8-Hydroxyquinoline–Thionyl Chloride–Ammonia Vapors

Vries, G., Brinkman, U.A.T.: "A Sensitive and Selective Reaction for Nitrate-Application in Thin-Layer Chromatography", *Mikrochim. Acta (Vienna)* 1984, 47–52.

Reagent Sequence for:

- Nitrate and nitrite ions

Preparation of the Reagents

Reagent 1 Coupling reagent

Dipping solution 1: Dissolve 500 mg 4-aminobenzenesulfonic acid and 150 mg 8-hydroxyquinoline in 100 ml of a mixture of acetone – diethyl ether – water (12 + 12 + 1). This solution is stable for several months. It should be topped up with diethyl ether from time to time to make up for evaporation losses.

Reagent 2 Reduction

Dipping solution 2: Dissolve 3 g thionyl chloride in 100 ml decane.

Reagent 3 Adjustment of pH

Ammonia vapor: Place 5 to 10 ml conc. ammonia solution in the free trough of a twin-trough chamber.

Reaction

Nitrates and nitrites are first reduced to nitrosyl chloride with thionyl chloride. The volatile nitrosyl chloride then reacts with 4-aminobenzenesulfonic acid to yield a diazonium salt that then couples with 8-hydroxyquinoline to form a colored azo compound. Hence, the coupling reagent is applied to the chromatogram first.

Method

The dried chromatogram is first dipped in reagent solution 1 for 1 s, dried briefly in a stream of cold air and then dipped in reagent solution 2 for 1 s. The TLC/HPTLC plate is then held upright on tissue paper to allow excess reagent to drain away; when the layer appears matt it is covered with a glass plate and kept at room temperature for 5 min. Afterwards it is dried in a stream of hot air and exposed to ammonia vapor.

Nitrate and nitrite ions yield orange-brown to magenta-red chromatogram zones on a pale yellow background immediately on treatment with ammonia; these zones are stable for days in an atmosphere of ammonia.

Note: When combined with thin-layer chromatographic separation the reagent provides a specific detection method for nitrate and nitrite. The color development is often completed within a few minutes on silica gel plates. In the absence of ammonia vapor traces of oxides of nitrogen in the laboratory atmosphere can slowly cause the background to become reddish-brown. The simultaneous presence of the following ions in the chromatogram zones interferes with the detection of nitrate/nitrite: I^- , IO_3^- , IO_4^- , MoO_4^{2-} and H_2PO_4^- .

The detection limits are slightly dependent on the layer involved, lying between 5 and 10 ng (RP 8, RP 18, silica gel 60) and 50 ng (cellulose, NH_2) per chromatogram zone. The detection of 20 ng nitrate is not prevented by a one hundred-fold excess of the following ions: NH_4^+ , Li^+ , Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Ba^{2+} , Zn^{2+} , Hg^{2+} , Al^{3+} , Sb^{3+} , Bi^{3+} , Zr^{3+} , Ag^+ , Ni^{2+} , Mn^{2+} , UO_2^{2+} , F^- , ClO_4^- , Br^- , CN^- , NCS^- , BO_3^{3-} , SiF_6^{2-} , AsO_4^{3-} , AsO_3^{3-} , SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, SO_4^{2-} , OH^- and CH_3COO^- . Colored ions such as Fe^{3+} , Cu^{2+} , Cr^{3+} etc. can, however, interfere.

The reagent can be employed on silica gel, kieselguhr, Si 50000, RP, NH_2 and cellulose layers.

***tert*-Butyl Hypochlorite– Potassium Iodide/Starch**

Purkayastha, R.: “Simultaneous Detection of the Residues of Atrazine and Linuron in Water, Soil, Plant and Animal Samples by Thin-Layer Chromatography”, *Internat. J. Environ. Anal. Chem.*: 1971, 1, 147–158.

Reagent Sequence for:

- *s*-Triazine and urea herbicides
e.g. atrazine, linuron

Preparation of the Reagents

Reagent 1 Chlorination

Spray solution 1: Mix 1 ml *tert*-butyl hypochlorite with 100 ml cyclohexane.

Reagent 2 Oxidation

Spray solution 2: Dissolve 1 g potassium iodide and 1 g soluble starch in 100 ml water.

Reaction

The *s*-triazines undergo chlorination at nitrogen to yield reactive N-chloro derivatives which oxidize iodide to iodine in the second step. This then forms an intense blue iodine-starch inclusion complex with starch.

Method

First spray the dried chromatogram homogeneously with reagent 1. Then remove excess reagent in a stream of cold air in the fume cupboard (ca. 30 min for silica gel and for aluminium oxide layers). Then spray the chromatogram lightly with reagent 2.

Urea herbicides give pale yellow and *s*-triazines blue chromatogram zones on a colorless background.

Note: The reagent sequence is a modification of the “chlorine–potassium iodide–starch” reagent.

The detection limits – dependent on the TLC layer – lie between 0.2 and 0.5 µg substance per chromatogram zone for atrazine and linuron.

The reagent can be employed on aluminium oxide, silica gel, kieselguhr and Si 50 layers.

***tert*-Butyl Hypochlorite– Potassium Iodide/*p*-Tolidine**

- [1] Halstrom, J., Brunfeldt, K., Thomsen, J., Kovács, K.: "Synthesis of the Protected C-Terminal Lys⁵-Heptapeptide of Eleodoisin by the Merrifield Method", *Acta Chem. Scand.* **1969**, *23*, 2335–2341.
- [2] Halstrom, J., Kovács, K., Brunfeldt, K.: "Synthesis of the N-Trityl Hexapeptide Hydrazide Corresponding to the Sequence 152–157 of the Coat Protein of Tobacco Mosaic Virus. Comparison of the Homogeneous and the Solid Phase Syntheses", *Acta Chem. Scand.* **1973**, *27*, 3085–3090.

Reagent Sequence for:

- Protected amino acids and peptides [1, 2]

Preparation of the Reagents

Reagent 1 Chlorination

Spray solution 1: *tert*-Butyl hypochlorite solution.

Reagent 2 Oxidation

Spray solution 2: Solution of potassium iodide and *p*-tolidine in glacial acetic acid/water.

Reaction

Reactive chloramine derivatives are produced in the first reaction step as a result of chlorination of the nitrogen by the *tert*-butyl hypochlorite; in the presence of potassium

iodide these derivatives oxidize *p*-tolidine to a deep blue semiquinonoid dye (cf. reagent monograph "chlorine – *o*-tolidine – potassium iodide").

Method

The dried chromatogram is evenly sprayed with the first and then with the second reagent.

Intensely colored chromatogram zones are produced on a colorless background.

Note: The reagent sequence can be employed on silica gel, kieselguhr and Si 50 layers.

***tert*-Butyl Hypochlorite– Potassium Iodide/*o*-Toluidine**

Heitz, W., Höcker, H., Kern, W., Ullner, H.: „Darstellung und Eigenschaften von linearen Oligourethanen aus Diethylenglykol und Hexamethyldiisocyanat“, *Makromol. Chem.* 1971, 150, 73–94.

Reagent Sequence for:

- Oligourethanes

Preparation of the Reagents

Reagent 1 Chlorination

tert-Butyl hypochlorite vapor: Place 10 ml *tert*-butyl hypochlorite in one half of a twin-trough chamber.

Reagent 2 Oxidation

Spray solution: Slowly mix a solution of 1.6 g *o*-toluidine in 30 ml glacial acetic acid with a solution of 2 g potassium iodide in 500 ml water.

Reaction

First there is chlorination of nitrogen to yield reactive N-chloro derivatives, which oxidize iodide to iodine in the next step. Finally oxidation of the *o*-toluidine probably yields colored quinonoid toluidine derivatives.

Method

The chromatogram is freed from mobile phase in the drying cupboard (10 min 160 °C) and placed while still hot in the chamber with *tert*-butyl hypochlorite vapor for 5 min. After removal of excess reagent (15 min stream of warm air) the chromatogram is sprayed with reagent 2.

This yields deeply colored chromatogram zones on a pale background.

Note: The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Danger warning: *o*-Toluidine is suspected of possessing carcinogenic properties. Therefore, the dipping method should be used if possible, if it is decided to use the spraying method in spite of this (cf. reagent monograph “Chlorine–*o*-toluidine reagent”).

Calcium Hypochlorite- Formaldehyde-Potassium Iodide/ Starch/Triton X-100

- [1] Schwarz, D.P., Sherman, J.T.: "Improved N-Chlorination Procedure for Detecting Amides, Amines and Related Compounds on Thin-Layer Chromatograms", *J. Chromatogr.* **1982**, *240*, 206-208.
- [2] Schwartz, D.P., McDonough, F.E.: "Practical Screening Procedure for Chloramphenicol in Milk at Low Parts per Billion Level", *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 563-565.

Reagent Sequence for:

- Amines, amino acids, amides [1, 2]
e.g. chloramphenicol, creatine, adenine, guanine
histidine, phenylalanine, sphingosine

Preparation of the Reagents

- Reagent 1 Chlorination**
Chlorine gas: Cover the base of a tightly sealable chromatographic trough with solid calcium hypochlorite to a depth of ca. 0.5 cm. This salt must be renewed weekly [1, 2].
- Reagent 2 Reduction of excess chlorine**
Formaldehyde gas: Fill one trough of a twin-trough chamber with 20 ml formalin solution (37%) [1, 2].
- Reagent 3 Oxidation and complex formation**
Spray solution: Dissolve 1 g potato starch, 1 g potassium iodide and 50 mg Triton X-100 in 100 ml water with warming [1].

Reaction

Primary and secondary amines and amides are first chlorinated at nitrogen by the chlorine released by the gradually decomposing calcium hypochlorite. Excess chlorine gas is then selectively reduced in the TLC layer by gaseous formaldehyde. The reactive chloramines produced in the chromatogram zones then oxidize iodide to iodine, which reacts with the starch to yield an intense blue iodine-starch inclusion complex.

Method

The chromatogram is dried in a stream of warm air and placed in the trough chamber with the calcium hypochlorite (reagent 1) for 2 min followed by 30-45 s in the free trough of a twin-trough chamber with the formalin solution (reagent 2). The chromatogram is then sprayed with reagent 3.

Intense blue-black chromatogram zones are produced on a colorless background.

Note: This reagent sequence is a modification of the reagent "chlorine - potassium iodide - starch". Mobile phases containing ammonia must be removed completely before treatment with the reagent sequence, since otherwise the background will be colored too. Some secondary amines (e.g. diphenylamine) and some amides (e.g. 2,4-dinitrobenzamide) and methionine sulfoxide do not give reactions even in quantities of up to 1 to 2 µg.

The detection limits for primary and secondary amines and for the amides are 10 to 60 ng substance per chromatogram zone [1]. The detection limits on layers with fluorescence indicators are about double the amount of substance [1].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Cerium(IV) Sulfate/Sodium Arsenite/ Sulfuric Acid–Methylene Blue– Ammonia Vapor

Naidoo, S.: "Separation of Acetic and Propionic Acid Analogs of L-Thyroxine and L-Triiodothyronine by Thin-Layer Chromatography", *Anal. Biochem.* 1978, 91, 543–547.

Reagent Sequence for:

- Iodide ions and organic iodine compounds
e.g. thyroxine, triiodo- and tetraiodothyronine

Preparation of the Reagents

Reagent 1	Redox reaction <i>Spray solution 1:</i> Mix solutions 1 and 2 in a volume ratio of 2 + 3 immediately before use. <i>Solution 1:</i> Dissolve 10 g cerium(IV) sulfate in 100 ml sulfuric acid (10%). <i>Solution 2:</i> Dissolve 5 g sodium metaarsenite in 100 ml water.
Reagent 2	Redox reaction <i>Spray solution 2:</i> Dissolve 50 mg methylene blue in 100 ml water.
Reagent 3	Neutralization of excess acid <i>Ammonia vapor:</i> Concentrated ammonia solution in one half of a twin-trough chamber.

Reaction

The course of the reaction has not been elucidated. Probably redox reactions involving cerium(IV) and arsenic(III) are catalyzed by iodide ions and organic iodine compounds with methylene blue acting as a redox indicator.

Method

The dried chromatogram is sprayed homogeneously first with spray solution 1 and then with reagent 2. Finally the chromatogram is exposed to an atmosphere of ammonia.

Iodides and organic iodine compounds produce brilliant blue chromatogram zones on a yellow background.

Note: The detection limits for iodides and organic iodine compounds are reported to be 50 to 100 ng substance per chromatogram zone.

The reagent can be employed on silica gel H layers.

Phosphoric Acid–Phosphomolybdic Acid

- [1] Mikac-Devic, D., Mišić, M., Stankovic, H.: "Quantitative Method for the Determination of 17-Oxosteroid Fractions by Thin-Layer Chromatography", *Z. Klin. Chem. Klin. Biochem.* 1970, 8, 361–363.
- [2] Detter, F., Kollmeier, W., Klingmüller, V.: „Das Muster der neutralen 17-Ketosteroide des Harns aufgrund der Dünnschicht-Chromatographie“, *Z. Klin. Chem. Klin. Biochem.* 1967, 5, 153–155.

Reagent Sequence for:

- 17-Oxosteroids (17-ketosteroids) [1, 2]
e.g. dihydroxyandrosterone, androsterone, aetiocholanolone, 11-oxoandrosterone

Preparation of the Reagents

Reagent 1 **pH adjustment**

Spray solution 1: Phosphoric acid (30%).

Reagent 2 **Redox reaction**

Spray solution 2: Alcoholic phosphomolybdic acid (10% in ethanol).

Reaction

In spite of the numerous publications the reaction mechanism is still not finally clarified. A large number of organic compounds can be oxidized by phosphomolybdic acid, with the reduction of some of the Mo^{VI} to Mo^{IV} , which then reacts with the re-

maining Mo^{VI} to yield a blue-gray mixed oxide (= molybdenum blue). The reduction of phosphomolybdic acid is pH-dependent.

Method

The dried chromatograms are first homogeneously sprayed with reagent 1 and then heated to 110°C for 10 min. After cooling to room temperature they are sprayed with reagent 2 and then heated again to 110°C for 10 min.

17-Oxosteroids produce blue-black chromatogram zones on colorless to pale yellow backgrounds.

Note: The derivatized steroids can be extracted from the blue chromatogram zones with alcohol and quantitatively determined by means of the ZIMMERMANN reaction, which is not interfered with by the presence of phosphoric acid and phosphomolybdic acid. A yellow background can be bleached by exposure to ammonia vapor [2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide– 4-Aminoantipyrine–Potassium Hexacyanoferrate(III)

Gosselé, J.A.W.: "Modified Thin-Layer Chromatographic Separation of Preservatives",
J. Chromatogr. 1971, 63, 433–437.

Reagent Sequence for:

- Antioxidants
e. g. 4-hydroxybenzoic acid and its esters

Preparation of the Reagents

Reagent 1	Hydrolysis <i>Spray solution 1:</i> Dissolve 10 g sodium hydroxide pellets in 100 ml water.
Reagent 2	Condensation <i>Spray solution 2:</i> Dissolve 2 g 4-aminoantipyrine in 100 ml ethanol.
Reagent 3	Oxidation <i>Spray solution 3:</i> Dissolve 8 g potassium hexacyanoferrate(III) in 100 ml water.

Reaction

When oxidized by iron(III) ions 4-aminoantipyrine reacts with phenols to yield color quinonoid derivatives (cf. 4-aminoantipyrine – potassium hexacyanoferrate(I) reagent in Volume 1 a). It is an oxidative coupling based on the EMERSON reaction

Method

The dried chromatogram is first sprayed homogeneously with spray solution 1 and then heated to 80°C for 5 min. After cooling to room temperature the TLC plate is sprayed with water and heated to 80°C for another 5 min, after which it is homogeneously lightly sprayed with reagent 2 and then with reagent 3.

Red to reddish-brown chromatogram zones are produced on a pale background.

Note: It is recommended that only small quantities of reagents be sprayed.

The detection limits lie between 0.25 and 1 µg substance per chromatogram zone. The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide-Cobalt(II) Acetate-*o*-Tolidine

Patil, V.B., Sevalkar, M.T., Padalikar, S.V.: "Thin-layer Chromatographic Detection of Endosulfan and Phosphamidon by Use of Cobalt Acetate and *o*-Tolidine", *J. Chromatogr.* **1990**, 519, 268-270.

Reagent Sequence for:

- Insecticides
e.g. endosulfan, phosphamidon

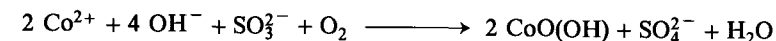
Preparation of the Reagents

- | | |
|------------------|-----------------------------------------------------------------------------------------------------------|
| Reagent 1 | Hydrolysis
<i>Spray solution 1:</i> Dissolve 5 g sodium hydroxide pellets in 100 ml water. |
| Reagent 2 | Oxidation
<i>Spray solution 2:</i> Dissolve 5 g cobalt(II) acetate in 100 ml water. |
| Reagent 3 | Oxidation
<i>Spray solution 3:</i> Dissolve 1 g <i>o</i> -tolidine in 100 ml acetic acid (10%). |

Reaction

The sulfite group present in the heterocyclic ring of endosulfan is rapidly hydrolyzed by alkali. In the presence of atmospheric oxygen it then oxidizes divalent cobalt to cobalt(III). Phosphamidon, which contains a 2-chloro-2-diethylcarbamoyl group, ox-

idizes divalent cobalt to trivalent, which then oxidizes *o*-tolidine to a blue-colored quinonoid derivative.



Method

Firstly, the dried chromatograms are homogeneously sprayed sequentially with solutions 1 and 2 and then, after being allowed to stand for 5 min, they are sprayed with reagent 3.

Intense blue chromatogram zones are produced on a colorless background; in medium they remain stable for ca. 30 min and then slowly fade.

Note: When the concentration of substance is sufficiently high endosulfan and phosphamidon sometimes appear as yellowish-brown zones after application of first two reagents of the sequence.

The reagent sequence is specific for endosulfan and phosphamidon. Other insecticides, e.g. organochlorine insecticides, such as endrin, aldrin, dieldrin, DDT, BHC, organophosphorus insecticides, such as malathion, parathion, dimethoate, quinalphos, phorate and fenitrothion, or carbamate insecticides, such as baygon, baryl and carbofuran do not react. Neither is there interference from amino acids, peptides or proteins which might be extracted from the biological material together with the pesticides.

Warning: The substances benzidine and *o*-dianisidine, which are classified as carcinogenic, react in a similar manner to *o*-tolidine, which is also suspected of causing cancer.

The detection limits are 1 µg substance per chromatogram zone (corresponding to 10 µg/g biological material).

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide–Iodine/ Potassium Iodide/ Sodium Azide–Starch

Vandamme, E.J., Voets, J.P.: "Separation and Detection of Degradation Products of Penicillin and Cephalosporins by Means of Thin-Layer Chromatography", *J. Chromatogr.* 1972, 71, 141–148.

Reagent Sequence for:

- Antibiotics with a thiazolidine ring
e.g. penicillins, cephalosporins and their degradation products
such as penicillin V, penicillin G, oxacillin, cloxacillin,
ampicillin, methicillin, cephalosporin C
- Sulfur-containing amino acids
e.g. cysteine

Preparation of the Reagents

- | | |
|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Reagent 1 | Hydrolytic ring opening
<i>Spray solution 1:</i> Sodium hydroxide (c = 2 mol/L). |
| Reagent 2 | Redox reaction (iodazide reaction)
<i>Spray solution 2:</i> Dissolve 1 g sodium azide in a solution of 127 mg iodine and 200 mg potassium iodide in 100 ml water. |
| Reagent 3 | Iodine starch complex formation
<i>Spray solution 3:</i> Dissolve 1 g soluble starch in 100 ml water. |

Reaction

Detection depends on the "iodine – azide reaction" which is normally extremely slow but is accelerated in the presence of divalent sulfur (cf. iodine-potassium chloride solution – sodium azide – starch reagent, AWE's reagent). The reaction involves the conversion of iodine to iodide, so that the iodine is no longer available for formation of the intense blue-colored iodine starch inclusion complex. The sodium hydroxide used as reagent 1 presumably acts to open the thioether linkage or the thiazolidine ring. Thiol and sulfhydryl groups accelerate the iodine-azide reaction appreciably more than the sulfur bound to the thiazolidine ring.

Method

The dried chromatogram is homogeneously sprayed successively with reagents 1, 2 and 3.

Pale chromatogram zones are produced on an intense blue-colored background.

Note: Sulfides, thiols and thioethers also react. The blue background of the chromatogram treated with the reagent sequence fades with time.

The detection limits for penicillin derivatives and cephalosporins are 0.5 to 1 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide- 4-Nitrobenzaldehyde- 1,2-Dinitrobenzene

Khazanchi, R., Handa, S.K.: "Detection and Separation of Fenpropathrin, Flucythrinate, Fluvalinate and PP 321 by Thin-Layer Chromatography", *J. Assoc. Off. Anal. Chem.* 1989, 72, 512-514.

Reagent Sequence for:

- Pyrethroids with α -cyano ester groups
e.g. fenpropathrin, flucythrinate, fluvalinate, PP 321

Preparation of the Reagents

Reagent 1 Hydrolysis

Spray solution 1: Dissolve 1 g sodium hydroxide in 5 ml water and make up to 50 ml with methanol.

Reagent 2 Cyanhydrin formation

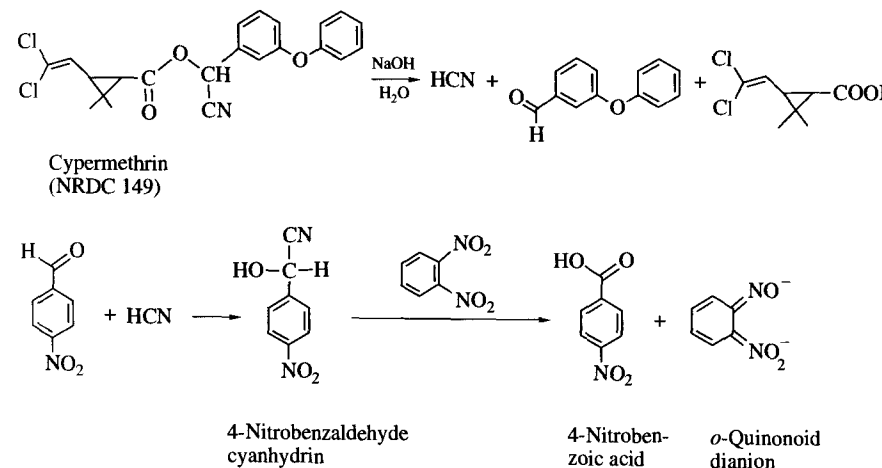
Spray solution 2: Dissolve 0.3 g 4-nitrobenzaldehyde in 10 ml 2-methoxyethanol (methylcellosolve).

Reagent 3 Formation of an *o*-quinoid di-anion

Spray solution 3: Dissolve 0.25 g 1,2-dinitrobenzene in 10 ml 2-methoxyethanol.

Reaction

Synthetic pyrethroids with α -cyano ester group react with sodium hydroxide to yield hydrogen cyanide, which reacts with 4-nitrobenzaldehyde and 1,2-dinitrobenzene to yield a pink-colored derivative (*o*-quinonoid di-anion) according to the following scheme:



Method

The chromatograms are dried in a stream of cold air and first sprayed homogeneousl with reagent 1 and allowed to stand for 3 min. Then they are sprayed with reagent followed immediately by reagent 3.

Pink-colored chromatogram zones are produced on a colorless background.

Note: Spray solutions 2 and 3 can be applied in any order or as a mixture.

The detection limits are 100 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Tin(II) Chloride– Ammonium Thiocyanate

Gaibakian, D.S., Rozylo, J.K., Janicka, M.: "Thin-Layer Chromatography Method for the Investigations of Re(VII), Mo(VI) and V(V) ions mixtures", *J. Liq. Chromatogr.* **1985**, *8*, 2969–2989.

Reagent Sequence for:

- Transition metal anions
e. g. Re^(VII), Mo^(VI), V^(V)

Preparation of the Reagents

Reagent 1 Reduction

Spray solution 1: Dissolve 10 g tin(II) chloride in 100 ml 6 mol hydrochloric acid.

Reagent 2 Complex formation

Spray solution 2: Dissolve 5 g ammonium thiocyanate in 10 ml water.

Reaction

The initial step of the reaction with tin(II) chloride reduces the highly oxidized metal in the transition metal anions to low valency cations; these are capable of forming stable colored complexes with thiocyanate.

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then after a short interval, with reagent 2.

Rhenium, molybdenum and vanadium ions yield orange, pink and yellow-colored chromatogram zones respectively on a colorless background.

Note: Iron(III) cations also react and give the well known deep red iron(III) isothiocyanate.

No details of the detection limits were provided.

The reagent can be employed on aluminium oxide, silica gel, kieselguhr, Si 5000 RP and cellulose layers.

Tin(II) Chloride- Borate Buffer-Fluorescamine

Haefelfinger, P.: "Determination of Nanogram Amounts of Primary Aromatic Amines and Nitro Compounds in Blood and Plasma", *J. Chromatogr.* 1975, *III*, 323-329.

Reagent Sequence for:

- Nitroaromatics
e. g. flunitrazepam

Preparation of the Reagents

Reagent 1 Reduction

Spray solution 1: Dissolve 4 g tin(II) chloride dihydrate in 100 ml acetic acid (5 %) and add 1 ml of a solution of 0.5 g phenolphthalein in 100 ml dioxane.

Reagent 2 pH adjustment

Spray solution 2A: Sodium hydroxide solution (2 %).
Spray solution 2B: Dissolve 31 g boric acid in 100 ml 1 mol sodium hydroxide solution and dilute with 800 ml water. Adjust the pH of this solution to 8.4 and make up to 1000 ml with water.

Reagent 3 Condensation

Spray solution 3: Dissolve 100 mg fluorescamine in 100 ml acetone.

Reaction

In the first step tin(II) chloride in acetic acid solution reduces the aromatic nitro group to amino groups. The aromatic amines produced then react with fluorescamine in a weakly basic medium to yield fluorescent derivatives (cf. reagent monograph "Fluorescamine Reagent", Volume 1a).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until the layer begins to become transparent and then heated to 105 to 110 °C for 10 min. After cooling to room temperature they are then sprayed with reagent 2A until the background is pale purple (color change of the pH indicator phenolphthalein) and then sprayed with reagent 2B until the layer begins to appear transparent. When the layer is evenly wetted it is then sprayed with reagent 3 and dried in a stream of warm air (ca. 70 °C).

On examination under long-wavelength UV light ($\lambda = 365$ nm) there are yellow fluorescent chromatogram zones on a dark background.

Note: The detection limits for flunitrazepam and its 7-nitrodesmethyl metabolites are 1 to 2 ng/ml plasma or 0.5 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Titanium(III) Chloride– 4-(Dimethylamino)-benzaldehyde

Suzuki, T., Uchiyama, M.: "Pathway of Nitro Reduction of Parathion by Spinach Homogenate", *J. Agric. Food Chem.* 1975, 23, 281–286.

Reagent Sequence for:

- Oxidized aromatic amines
e.g. metabolites of parathion

Preparation of the Reagents

Reagent 1 Reduction

Spray solution 1: Dissolve 0.5 g titanium(III) chloride in 100 ml N hydrochloric acid.

Reagent 2 Condensation

Spray solution 2: Dissolve 0.5 g 4-(dimethylamino)-benzaldehyde in a mixture of 50 ml ethanol and 50 ml glacial acetic acid.

Reaction

In a first step oxidized aromatic amines are reduced with titanium(III) chloride in glacial acetic acid solution and then condensed to a colored SCHIFF's base with 4-(dimethylamino)-benzaldehyde (cf. Chapter 2).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then after an interval of a few minutes, with reagent 2.

The oxidized aromatic amines yield yellow-colored chromatogram zones on a colorless background.

Note: The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

3.3 Azo Coupling

Coupling reactions with diazonium salts to yield intensely colored azo derivatives have often been used for the detection of phenols, primary aromatic amines and electron-rich heterocyclics.

These reactions can be opened up to all substances that can yield aniline derivatives in acid or basic medium. Carbamic acid derivatives, numerous variations of which are used as plant treatment agents, provide a striking application. As do urea herbicides and a variety of drug substances e.g. benzodiazepines or phenylbutazone derivatives.

The aromatic nitro compounds make up another group of substances. These can also be formed directly on the TLC layer as a result of the frequently used VITALI reaction [24]. They are detected — as shown in Fig. 21 — by reduction and coupling to azo dyes.

The examples reproduced below have been taken from the literature.

Hydrochloric Acid Vapor— Sodium Nitrite/Hydrochloric Acid— Amidosulfonic Acid— N-(1-Naphthyl)-ethylenediamine

Bernhard, W., Jeger, A.N., De La Vigne, U.: "Identification and determination of hydrochlorothiazide [Esidrix®]", *J. Planar Chromatogr.* 1989, 2, 77-79.

Reagent Sequence for:

- Diuretics
e.g. hydrochlorothiazide (Esidrix®)

Preparation of the Reagents

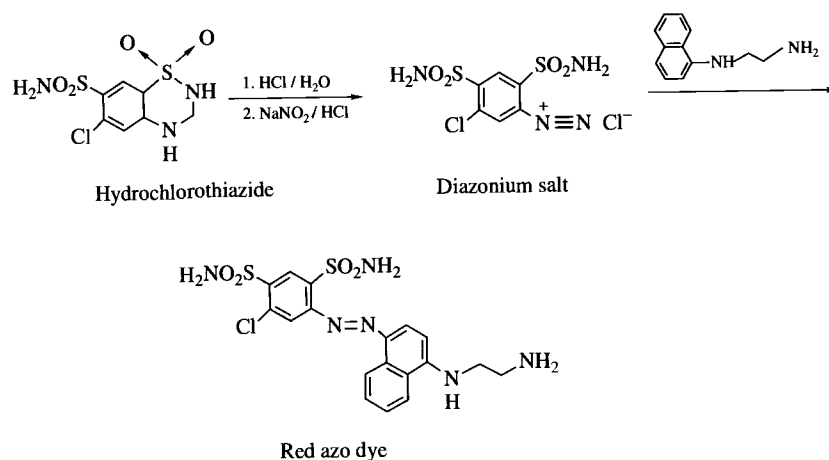
Reagent 1	Hydrolysis <i>Hydrochloric acid vapor:</i> This can be generated by placing 10 ml of concentrated hydrochloric acid (37%) in one trough of a twin-trough chamber.
Reagent 2	Diazotization <i>Spray solution 1:</i> Dissolve 4 g sodium nitrite in 40 ml water. The solution is stable over a long period. <i>Spray solution 2:</i> Hydrochloric acid (10%).
Reagent 3	Destruction of excess nitrite <i>Spray solution 3:</i> Dissolve 500 mg ammonium amidosulfonic acid (amidosulfonic acid ammonium salt) in 100 ml water.

Reagent 4 Coupling

Spray solution 4: Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml ethanol. The solution should always be made up fresh even though it remains stable for several days.

Reaction

In the first reaction step the heterocyclic ring is opened by hydrolysis to yield a primary amino group, that is then diazotized and finally coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye.

**Method**

The dried chromatograms are first exposed to hydrochloric acid vapor (reagent 1) for a few minutes and then heated to 120 °C for 5 min to remove excess hydrochloric acid. After cooling to room temperature the TLC/HPTLC plates are successively sprayed homogeneously with spray solutions 1 and 2. They are then dried briefly in a stream

of cold air, sprayed with reagent 3, dried once again in a stream of cold air and finally sprayed with reagent 4 and dried in a stream of warm air.

Red chromatogram zones are produced on a light background.

Note: When this reagent sequence is combined with the in situ measurement of the UV spectra of the chromatogram zones before derivatization this reagent sequence becomes virtually specific for hydrochlorothiazide and its related derivatives in the analysis of urine. Hence, it is possible to use it for drug monitoring and doping control.

The detection and determination limits are less than 0.1 and 0.2 mg hydrochlorothiazide per liter body fluid and thus appreciably lower than the therapeutic levels which are reported to be between 0.2 and 1.6 mg/l.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Iodine-Sodium Carbonate-Sulfanilic Acid, Diazotized

- [1] Ritter, W., Plempel, M., Pütter, J.: „Drei Methoden zur Konzentrationsbestimmung von Clotrimazol in biologischem Material“, *Arzneim. Forsch.* 1974, 24, 521-525.
 [2] Ritter, W.: “Post-Chromatographic Derivatizations in Quantitative (HP)TLC”, *Proc. 2nd Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr. (HPTLC)* p. 100-113, Interlaken, 1982.

Reagent Sequence for:

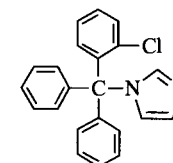
- Imidazole antimycotics
 e.g. clotrimazole [1, 2]

Preparation of the Reagents

Reagent 1	Cleavage by iodine <i>Spray solution 1:</i> Dissolve 1 g iodine in 100 ml ethanol.
Reagent 2	pH adjustment <i>Spray solution 2:</i> Dissolve 20 g sodium carbonate in water and make up to 100 ml.
Reagent 3	Coupling with PAULY's reagent <i>Solution A:</i> Dissolve 0.3 g sulfanilic acid in 100 ml 10% hydrochloric acid. <i>Solution B:</i> Dissolve 10 g sodium nitrite in water and make up to 100 ml. <i>Spray solution 3:</i> Mix 8 parts by volume solution A with 2 parts by volume solution B immediately before use; this mixture can be used for about 1 h.

Reaction

The mechanism of the reaction has not been elucidated. Presumably iodine eliminates the imidazole ring from N-substituted imidazole derivatives such as clotrimazole, and this then couples with diazotized sulfanilic acid to yield an azo dye.



Clotrimazole (Bay b 5097)

Method

The dried chromatograms are sprayed homogeneously with reagent 1, then heated to 100 °C in a vacuum drying cupboard, first at atmospheric pressure for 15-20 min and then under vacuum for a further 5-10 min (removal of excess iodine). After cooling to room temperature the chromatograms are sprayed with reagent solution 2 and then dried at 100 °C (atmospheric pressure). The chromatograms are finally sprayed with reagent solution 3 and dried at 100 °C [1, 2].

Red to violet chromatogram zones, that can be recorded photometrically $\lambda = 530$ nm, are produced on a colorless background.

Note: The reaction is very specific for N-substituted imidazole derivatives. In serum investigations the detection limit was 50 ng clotrimazole per milliliter serum. The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Nitric Acid–Sodium Dithionite– Sodium Nitrite–N-(1-Naphthyl)- ethylenediamine

Haefelfinger, P.: "Determination of Amitriptyline and Nortriptyline in Human Plasma by Quantitative Thin-Layer Chromatography", *J. Chromatogr.* **1978**, *145*, 445–451.

Reagent Sequence for:

- Aromatic substances
e. g. antidepressives
such as amitriptyline, nortriptyline

Preparation of the Reagents

- | | |
|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Reagent 1 | Nitration
<i>Spray solution 1:</i> Mix equal volumes of nitric acid (65%) and methanol while cooling with ice. The reagent solution may be kept for several weeks. |
| Reagent 2 | Reduction
<i>Spray solution 2:</i> Dissolve 4 g sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in 100 ml 0.5 mol phosphate buffer solution (pH 6.5). This solution is only stable for about 1 h, so it should always be made up fresh. |
| Reagent 3 | Diazotization
<i>Spray solution 3:</i> Dissolve 400 mg sodium nitrite in 20 ml 1 mol hydrochloric acid solution. The solution should always be made up fresh. |

Reagent 4 Coupling

Spray solution 4: Dissolve 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml methanol. This solution should be made fresh, even though it is reported to be stable for several days.

Reaction

The first step of the reaction involves nitration of the aromatic skeleton of the substance to be detected. Then the aromatic nitro derivatives so produced are reduced with sodium dithionite, in acid medium, to the corresponding amines; these are then diazotized and coupled with N-(1-naphthyl)-ethylenediamine to yield an azo compound (cf. Fig. 21).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until the layer begins to become transparent and are then heated to 125–130 °C for 15 min. After cooling to room temperature the TLC/HPTLC plates are homogeneously sprayed with reagent solution 2 and heated to 80–85 °C for 8 min. After cooling to room temperature the plates are sprayed homogeneously with reagent 3 and then thoroughly dried (10 min) in a stream of cold air. Finally the chromatograms are sprayed with reagent 4 and then dried in a stream of warm air for 1 min.

Reddish chromatogram zones are formed on a light background.

Note: Derivatization with this reagent sequence in combination with extraction and TLC separation is specific for amitriptyline and nortriptyline in the analysis of plasma; furthermore its high sensitivity allows its employment in pharmacokinetic studies, e.g. after the oral administration of a single dose of 25 mg amitriptyline.

The plate should be heated as rapidly and as evenly as possible after the first spray step; this is best done on a thick preheated aluminium plate in a drying cupboard equipped with ventilation allowing the nitric acid vapors to be removed using a water pump or vacuum. Sodium dithionite is better than titanium(III) chloride or tin(II) chloride for the reduction of the nitro derivatives of amitriptyline and nortriptyline.

The amino derivatives of amitriptyline and nortriptyline produced at the second heating stage exhibit intense pale yellow fluorescence on examination in long-wave-length UV light ($\lambda = 365$ nm), but this is not sufficiently reproducible for quantitative in situ work.

The detection limits for amitriptyline and nortriptyline are ca. 500 pg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Nitric Acid/Sulfuric Acid-Titanium(III) Chloride-Sodium Nitrite-N-(1-Naphthyl)-ethylenediamine

- [1] Haefelfinger, P.: "Determination of Nanogram Amounts of Aromatic Compounds by Spectrophotometry on Thin-Layer Chromatograms", *J. Chromatogr.* **1976**, *124*, 351-358.
- [2] Albet, C., Sánchez, M.G., Colomé, J.: "Determination of a new mucolytic drug Adamexina in biological liquids by photodensitometry", *J. Chromatogr.* **1980**, *181*, 504-511.

Reagent Sequence for:

- Aromatic substances
 - e.g. antitussives [1]
 - such as chlorpheniramine, codeine
 - e.g. mucolytics [2]
 - such as adamexine, bromexine

Preparation of the Reagents

Reagent 1

Nitration

Spray solution 1: Cautiously and with cooling mix 30 ml nitric acid (65%) with 10 ml sulfuric acid and add the mixture with cooling to 40 ml methanol. The reagent solution may be kept several weeks.

Reagent 2 Reduction

Spray solution 2: Dilute 4 ml of a solution of 15 g titanium(III) chloride in 100 ml hydrochloric acid (4%) to 20 ml with methanol. This solution is only stable for about 1 h and should, therefore, always be made up fresh.

Reagent 3 Diazotization

Spray solution 3: Dissolve 400 mg sodium nitrite in 20 ml 1 mol hydrochloric acid. This solution should always be made up fresh.

Reagent 4 Coupling

Spray solution 4: Dissolve 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml methanol. The solution should always be made up fresh even though it remains stable for several days.

Reaction

The aromatic skeleton is nitrated in the first reaction step. Then titanium(III) chloride in acid medium is used to reduce the aromatic nitro compounds so produced to the corresponding amines, which in turn are diazotized and coupled to N-(1-naphthyl)-ethylenediamine to yield an azo dye (cf. Fig. 21).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until they start to become transparent and then heated to 110–115 °C for 10–15 min. After cooling to room temperature the TLC/HPTLC plates are sprayed with reagent 2 and heated to 110–115 °C for 5 to 30 min. The layers are cooled to room temperature, sprayed with reagent 3 and then dried in a stream of cold air for 10 min. Finally the chromatograms are treated with reagent 4 and dried in a stream of warm air (ca. 50–60 °C) for 1 min.

Chlorpheniramine produces purple zones and codeine bluish-gray zones on a light background [1]. Adamexine and bromexine produce pinkish-violet zones, that are stable for ca 24 h in the dark, on a yellowish-white background [2].

Note: Derivatization with this sequence of reagents in combination with extraction and TLC separation is virtually specific for chlorpheniramine in the analysis of plasma [1];

its high sensitivity also makes it suitable for pharmacokinetic studies after the oral administration of a single therapeutic dose. If excited with long-wavelength UV light ($\lambda_{\text{exc}} = 365 \text{ nm}$) after the first reaction step codeine produces a fluorescent emission ($\lambda_{\text{fl}} > 578 \text{ nm}$) which can be used for quantitative measurement.

The plate should be heated as rapidly and homogeneously as possible after the first spraying step; this is best done on a thick preheated aluminium plate in a drying cupboard equipped with ventilation allowing the nitric acid vapors to be removed using a water pump vacuum [1].

The detection limits are 1 to 2 ng/ml plasma for chlorpheniramine [1] and 50 and 250 ng per chromatogram zone for adamexine and bromexine respectively [2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Nitrous Fumes–N-(1-Naphthyl)-ethylenediamine

- [1] Singh, A.K., Ashraf, M., Granley, K., Mishra, U., Rao, M.M., Gordon, B.: "Screening and Confirmation of Drugs in Horse Urine by Using a Simple Column Extraction Procedure", *J. Chromatogr.* 1989, 473, 215–226.
- [2] Heuser, E., Reusche, W., Wrabetz, K., Fauss, R.: „Zur Bestimmung von freien, monomeren Toluylendiisocyanaten und Hexamethylenendiisocyanat in Polyurethan (=PUR)-Anstrichstoffen“, *Fresenius Z. Anal. Chem.* 1971, 257, 119–125.

Reagent Sequence for:

- Aromatic amines
e.g. clenbuterol [1]
N-methyl-N-(4-aminobenzyl)-amino derivatives of isocyanates [2]

Preparation of the Reagents

Reagent 1 Diazotization

Nitrous fumes: Nitrous fumes can be generated in one trough of a twin-trough chamber by mixing a solution of 1 g sodium nitrite in 5 ml water with 5 ml fuming hydrochloric acid.

Reagent 2 Coupling

Spray solution: Dissolve 10 mg N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml ethanol [1]. Alternatively it is possible to use 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml dimethylformamide – hydrochloric acid (c = 1mol/L) (1+1) [2].

Reaction

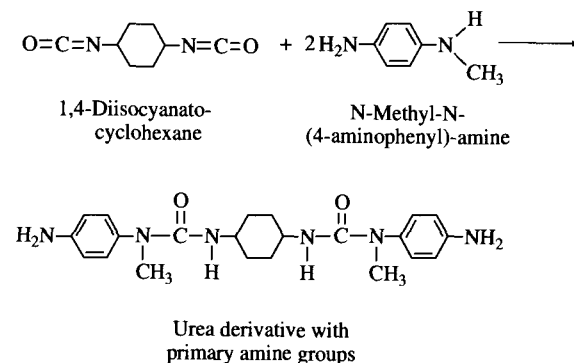
The aromatic amino group is diazotized in the first reaction step. The diazonium compound so formed is then coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye.

Method

The dried chromatograms are first treated with nitrous fumes for about 5 min. The excess nitrous fumes are then removed in a stream of cold air (ca. 5 min) and the TLC/HPTLC plates are sprayed homogeneously with reagent 2.

Clenbuterol, for example, yields pink-colored chromatogram zones on a light background.

Note: This reagent is a modification of the BRATTON-MARSHALL reagent. Isocyanate can be made accessible to this modification of the BRATTON-MARSHALL reagent by reacting prechromatographically with N-methyl-N-(4-aminophenyl)-amine to give the corresponding urea derivatives with primary aromatic amino groups:



This combination of the modified BRATTON-MARSHALL reaction with prechromatographic derivatization with N-methyl-N-(4-aminophenyl)-amine allows specific detection of isocyanates, that is especially applicable to aliphatic isocyanates.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Tin(II) Chloride–Sodium Nitrite– 1-Naphthol

- [1] Pugge, H.: Dissertation, Universität des Saarlandes, Saarbrücken, in preparation.
[2] Pugge, H., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1992.

Reagent Sequence for:

- Aromatic nitro compounds [1]
 - e.g. herbicides
 - such as trifluralin, pendimethalin
 - e.g. fungicides
 - such as dinocap

Preparation of the Reagents

Reagent 1	Reduction <i>Dipping solution 1:</i> Dissolve 2 g tin(II) chloride dihydrate in 20 ml hydrochloric acid (25%) and dilute with 30 ml methanol. The reagent should always be made up fresh.
Reagent 2	Diazotization <i>Nitrous fumes:</i> Mix a solution of 1 g sodium nitrite in 5 ml water with 5 ml fuming hydrochloric acid in one trough of a twin-trough chamber to generate nitrous fumes. The reagent should always be made up fresh.
Reagent 3	Coupling <i>Dipping solution 2:</i> Dissolve 1 g 1-naphthol in 100 ml ethanol. The solution may be stored in the refrigerator for several weeks.

Reaction

Aromatic nitro compounds are reduced to the corresponding amines by tin(II) chloride in acidic medium. These are then diazotized via the gas phase with nitrous fumes and finally coupled with 1-naphthol to yield an azo dye.

Method

The chromatograms are dried in a stream of cold air and then dipped in reagent 1 for 2 s, dried in a stream of cold air for 10 min and then cooled to -20°C for 15 min. The cold chromatogram is then placed in the free trough of a twin-trough chamber containing reagent 2 for 10 min for the purpose of diazotization via the gas phase. After removal of excess nitrous fumes by exposure to a stream of cold air for 5 min, the chromatograms are dipped in reagent 3 for 2 s and dried in a stream of cold air for 5 min.

Chromatogram zones of various colors are produced on a colorless background.

Note: The treated chromatogram should not be exposed to UV light or heat, neither should it be covered with a glass plate, since this causes it to turn dark brown in a short period of time. Nitrous fumes are corrosive so the diazotization and azo coupling should be carried out in the fume cupboard (rubber gloves).

The selectivity of the detection reaction can be increased by exposing the chromatogram to ammonia vapor after it has been treated with reagents 1 to 3; this can be done by placing 10 ml ammonia solution (25%) in the free trough of a twin-trough chamber. The result in general is that all chromatogram zones acquire a red to yellow-brown color, with the detection sensitivity of some substances being increased, while it can be reduced for others.

The color shades of the chromatogram zones and above all the pale background produced by this technique are stabilized by dipping the chromatogram in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s. The color shades produced on silica gel and RP layers are not identical.

The detection limits for 2,6-dinitroaniline herbicides are between 20 and 200 ng substance per chromatogram zone (Table 1). Similar results are also obtained with methyl and ethyl parathion (pink-colored zones).

The reagent can be employed on silica gel, kieselguhr, Si 50000 and RP layers.

Table 1: Absorption maxima and detection limits for some 2,6-dinitroaniline herbicides on silica gel layers after treatment with the reagent sequence and after additional exposure to ammonia vapor.

Substance	Without ammonia vapor		With ammonia vapor	
	λ_{\max} [nm]	Detection limits [ng]	λ_{\max} [nm]	Detection limits [ng]
Oryzalin	455	20	485	20
Nitralin	444	20	481	20
Dinitramin	440	200	452	100
Pendimethalin	583	5	496	20
Butralin	572	5	504	20
Fluchloralin	498	20	498	20
Isopropalin	437	20	441	20
Trifluralin	448	40	436	50

Procedure Tested

2,6-Dinitroaniline Herbicides [2]

Method	Ascending, one-dimensional double development in a twin-trough chamber without chamber saturation and with 10 min drying (cold air stream) after the first run.
Layer	HPTLC plates Silica gel 60 (MERCK), that were prewashed by immersing them in 2-propanol overnight and then drying at 110°C for 60 min. Immediately before the first development the HPTLC plates were conditioned for 20 min at 30% relative humidity, e. g. over sulfuric acid (50%, g/g).
Mobile phase	1. <i>n</i> -Hexane – toluene (17+10) 2. Toluene – methanol (85+15)
Migration distance	1. 8.5 cm 2. 1.5 cm
Running time	1. 35 min 2. 3 min

Detection and result: The chromatogram was dried for 10 min in a stream of cold air and dipped in reagent 1 for 2 s and dried for 10 min in a stream of cold air. The yellow

2,6-dinitroaniline compounds were converted to colorless derivatives by this process. The chromatogram was then cooled to -20°C for 15 min and exposed – still cold – to nitrous fumes for 10 min for diazotization; these fumes were generated in the empty trough of a twin-trough chamber by mixing 5 ml aqueous sodium nitrite solution (20%) with 3 to 5 ml fuming hydrochloric acid. Excess nitrous fumes were removed by exposing the layer for 5 min to a stream of cold air and the chromatogram was dipped in reagent 3 for 2 s and dried in a stream of cold air for 5 min.

The chromatogram zones produced were brown for oryzalin (migration distance 4–6 mm) and nitralin (10–15 mm), yellowish-brown for dinitramin (18–22 mm) and isopropalin (58–62 mm), blue for pendimethalin (38–42 mm), violet for butralin (43–48 mm), red for fluchloralin (50–55 mm) and orange for trifluralin (65–70 mm) the background was colorless (Fig. 1A).

These chromatograms acquired a dark brown coloration within seconds if they were heated or exposed to UV light; the same effect was observed on covering the chromatogram with a glass plate!

The chromatogram was then placed in a twin-trough chamber with ca. 10 ml ammonia solution (25%) to increase the sensitivity. Afterwards the chromatogram zones were red in the case of oryzalin, nitralin, dinitramin, pendimethalin, butralin and fluchloralin and yellowish-brown in the case of isopropalin and trifluralin (Fig. 1B). The detection sensitivity was sometimes increased and sometimes decreased (Table 1).

The colors obtained in this manner, and above all the pale background, were stabilized by dipping the chromatogram finally in a solution of liquid paraffin – *n*-hexane (1+2).

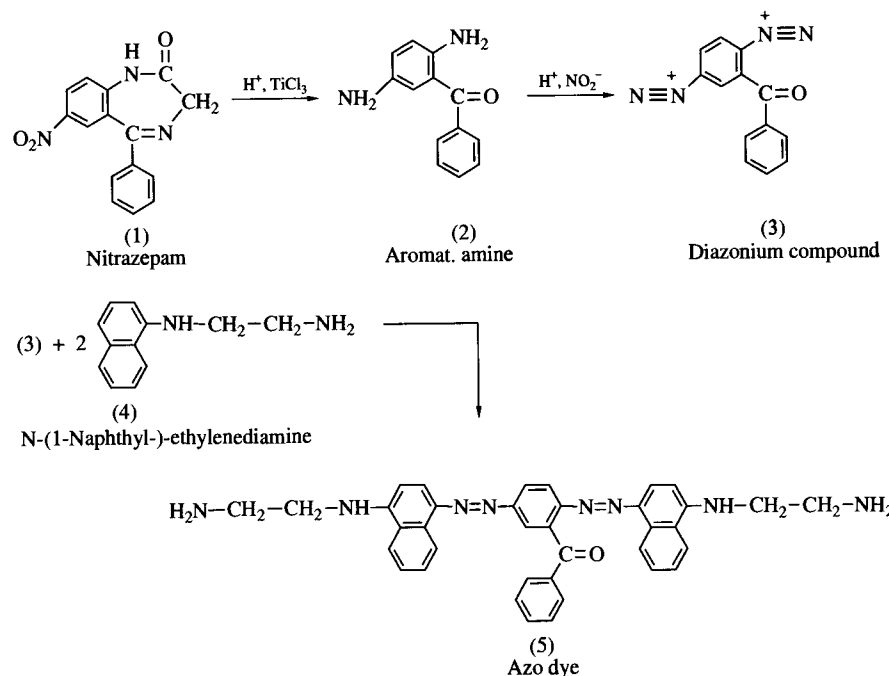
The detection limits are 20–200 ng substance per chromatogram zone (cf. Table 1).

In situ quantitation: The adsorption photometric evaluation in reflectance, before exposure of the chromatograms to ammonia, was carried out at $\lambda = 460$ nm (Fig. IIA) and $\lambda = 580$ nm (Fig. IIB), that after ammonia treatment at $\lambda = 490$ nm (Fig. IIC).

Fig. II: Reflectance scans of a chromatogram track with 200 ng g each of oryzalin (1), nitratin (2), pendimethalin (4), butralin (5), fluchloralin (6), isopropalin (7), trifluralin (8) and of 1000 ng dinitramin (3), per chromatogram zone; measurement at $\lambda = 460$ nm (A), 580 nm (B) and 490 nm (C); x = dipping fronts.

Reaction

Titanium(III) chloride (particularly in slightly alkaline medium) reduces the *p*-nitro groups of the thiophosphate insecticides to amino groups, which are then reacted with nitrite in acid medium in a second step to yield a diazonium compound as intermediate. This is then coupled to N-(1-naphthyl)-ethylenediamine dihydrochloride to yield an azo dye [3]. In the case of benzodiazepines the first reaction step includes an additional acid hydrolysis to the corresponding benzophenone derivative [2].



Method

After the chromatograms have been freed from mobile phase in a stream of warm air for 3 min they are immersed in dipping solution 1 for 3 s or homogeneously sprayed with it and then dried in a stream of warm air. Then they are dipped in reagent solu-

tion 2 for 3 s or homogeneously sprayed with it and finally, after drying in a stream of warm air, they are dipped in reagent solution 3 for 3 s or homogeneously sprayed with it.

Thiophosphoric acid insecticides and benzodiazepines yield reddish to bluish-violet-colored chromatogram zones on a colorless background.

Note: The reagent sequence can also be deployed in two stages with an intermediate chromatographic development using the SRS technique (separation – reaction – separation) [2]. When carrying out the acidic, reducing hydrolysis of benzodiazepines it is recommended that, after treatment with titanium(III) chloride in hydrochloric acid, the TLC plate be covered with a glass plate and heated to 100°C for ca. 10 min; afterwards the amines that have been formed can be released for subsequent separation by exposing the plate to ammonia vapor [2].

The diazotization reaction can also be initiated via the vapor phase, e.g. with ethyl nitrite that can be generated in one trough of a twin-trough chamber by adding a few drops of conc. hydrochloric acid to a mixture of ethanol and saturated aqueous sodium nitrite solution (1+1) [3]; the less volatile amyl nitrite can be used as an alternative [3].

The detection limits for thiophosphoric acid insecticides are 100 ng and for benzodiazepines 20 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr, Si 50000, RP and cellulose layers.

Procedure Tested

Organophosphorus Insecticides [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ extra thin layer (MERCK).
Mobile phase	<i>n</i> -Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (909+40+26+25+1)
Migration distance	8 cm
Running time	28 min

Detection and result: The developed chromatogram was dried for 3 min in a stream of warm air and first immersed for 3 s in dipping solution 1 and dried in a stream of warm

air. It was then immersed in dipping solution 2 for 3 s and finally treated with dipping solution 3.

Azinphos methyl (hR_f : 15–20), azinphos ethyl (hR_f : 20–25, parathion methyl (hR_f : 40–45), fenitrothion (hR_f : 45–50), parathion ethyl (hR_f : 60–65) and phoxim (hR_f : 60–65) appear as red-colored chromatogram zones on a colorless background.

The detection limits are 80–100 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 550$ nm (Fig. 1).

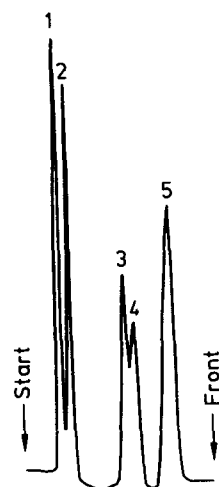


Fig. 1: Reflectance scan of a chromatogram track with 300 ng substance per chromatogram zone: 1 = azinphos methyl, 2 = azinphos ethyl, 3 = parathion methyl, 4 = fenitrothion, 5 = parathion ethyl + phoxim.

Titanium(III) Chloride– Nitrous Fumes– N-(1-Naphthyl)-ethylenediamine

VDI Richtlinie 2467: VDI-Handbuch „Reinhaltung der Luft“, Vol. 3, Beuth Verlag, Berlin.

Reagent Sequence for:

- Aromatic nitro compounds
e. g. dinitrophenyl derivatives of primary and secondary amines

Preparation of the Reagents

Reagent 1	Reduction <i>Spray solution:</i> Dilute 1 ml titanium(III) chloride solution ($c = \text{ca. } 15\%$ in hydrochloric acid (10%)) to 10 ml with hydrochloric acid (20%) or sulfuric acid (20%) and mix with 10 ml pyridine and 5 ml glacial acetic acid.
Reagent 2	Diazotization <i>Nitrous fumes:</i> These can be generated in one trough of a twin-trough chamber by mixing a solution of 1 g sodium nitrite in 5 ml water with 5 ml fuming hydrochloric acid.
Reagent 3	Coupling <i>Spray solution:</i> Dissolve 0.5 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml water, containing 2 drops conc. hydrochloric acid.

Reaction

The dinitrophenyl derivatives are reduced by titanium(III) chloride in acidic medium in the first step of the reaction to the corresponding aromatic amines; these are then diazotized and coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye (cf. Fig. 21).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1, then dried for 2 to 3 min in the air and for ca. 10 min at 60°C in a drying cupboard until the pyridine has completely evaporated. Afterwards they are briefly sprayed with a little hydrochloric acid (25%) and exposed to nitrous fumes (reagent 2) for several minutes. Finally after removal of the excess nitrous fumes in a stream of cold air the chromatograms are sprayed with reagent 3.

Blue-violet chromatogram zones are produced immediately on a pale background.

Note: This reagent sequence can be employed to great advantage after the prechromatographic derivatization of primary and secondary amines with 2,4-dinitrofluorobenzene; this makes for virtually specific detection of aliphatic amines.

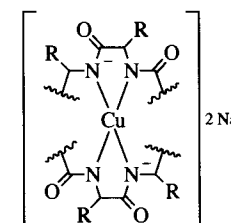
The detection limits for the aliphatic amines methyl, ethyl and isopropylamine are a few nanograms substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

3.4 Metal Complexes

The cations of some transition metals are electron acceptors that are capable of complexing with colorless organic compounds having electron donor properties. Color complexes are formed as a result of changes occurring in the electron orbitals of the central metal atom [4]. The most important of these chelate formers are Cu^{2+} , Fe^{3+} and Co^{2+} ions, which have a great affinity for compounds containing oxygen and nitrogen.

The frequently used biuret reaction of proteins results in the formation of the following reddish-violet complex in alkaline medium [4, 25, 26]:



Aromatic ethanolamine derivatives, e.g. ephedrine, also yield blue-colored chelates with Cu^{2+} ions (CHEN-KAO reaction [4, 27]).

Mono- and polyhydric phenols and enols frequently form characteristically colored complexes with Fe^{3+} ions [4, 28, 29]. Here monohydric phenols usually produce reddish-violet colors, while pyrocatechol derivatives yield green chelates [4]. Detection of acetone using LEGAL's test is based on the formation of an iron complex [4]. The same applies to the thioglycolic acid reaction of the German Pharmacopoeia (DAB [4, 30]).

The ZWIKKER reaction involving Co^{2+} salts is frequently used for the detection of barbituric acid derivatives [31–35], but some purine, pyridine and piperidine derivatives and heterocyclic sulfonamides also yield colored derivatives. The ZWIKKER reaction is particularly sensitive when it is possible to form a tetrahedral complex $[\text{Co}(\text{Barb})_2\text{X}_2]$ (X = donor ligand, e.g. amine) [4].

Hydroxylamine–Iron(III) Chloride

Schwarzmaier, U.: „Hydroxylamin/Eisen(III)-chlorid, ein stereospezifisches Farbreagenz für Mandelonitrilglycoside“, *J. Chromatogr.* 1975, III, 430–433.

Reagent Sequence for:

- Mandelonitrile glycosides (cyanoglycosides)
e.g. amygdalin, prunasin, taxiphyllin, vicianin

Preparation of the Reagents

Reagent 1 Nucleophilic substitution

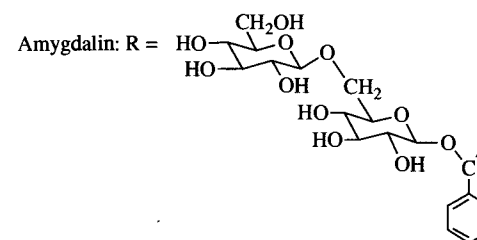
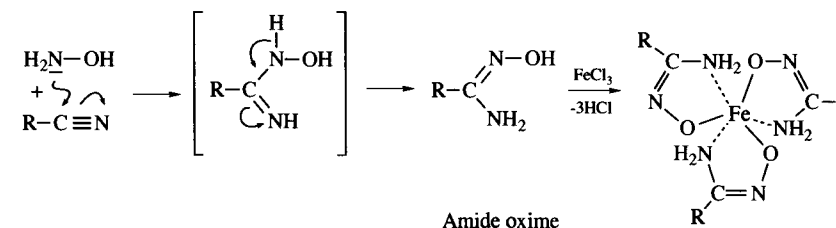
Spray solution 1: Add 9.5 g finely ground anhydrous potassium carbonate portionwise to an ice-cooled solution of 14 g hydroxylamine hydrochloride in 20 ml water. When the evolution of CO₂ gas has stopped add 80 ml ethanol. Stir for a further 30 min at 0°C and filter. The spray solution 1 that is produced has a pH of 5.0 to 5.5 and may be stored in the refrigerator for ca. 10 days.

Reagent 2 Complex formation

Spray solution 2: Dissolve 1.5 g iron(III) chloride in 100 ml methanol.

Reaction

Firstly there is nucleophilic attack of the nitrile carbon atom by hydroxylamine. An amide oxime is produced; this then forms an intensely colored complex with the iron(III) chloride.



Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then allowed to stand at room temperature for 15 min. They are then sprayed homogeneously with reagent 2.

Mandelonitrile glycosides with D-configuration (e.g. prunasin, taxiphyllin, *p*-O-methyltaxiphyllin, amygdalin vicianin) immediately yield reddish-brown chromatogram zones on a colorless background. L-isomers (e.g. sambunigrin, dhurrin, *p*-O-methyl-dhurrin, neoamygdalin) gradually yield chromatogram zones that are pale brown initially.

Note: The colors of the chromatogram zones produced by the mandelonitrile glycosides change with time: Those of the D-isomers turn steel blue within an hour, while the L-isomers turn reddish-brown during this time. It is possible to distinguish between D- and L-forms on the basis of these typical color differences and color changes. Both isomeric forms have a dark brown color after about 24 hours.

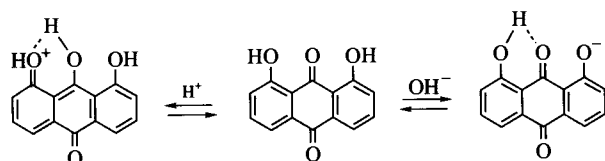
The detection limits for mandelonitrile glycosides are 3 to 5 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

3.5 Halochromism and Charge-Transfer Complexes

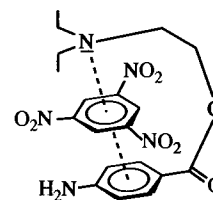
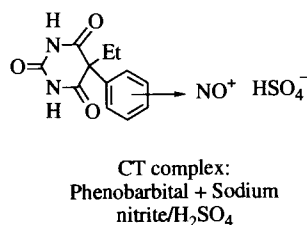
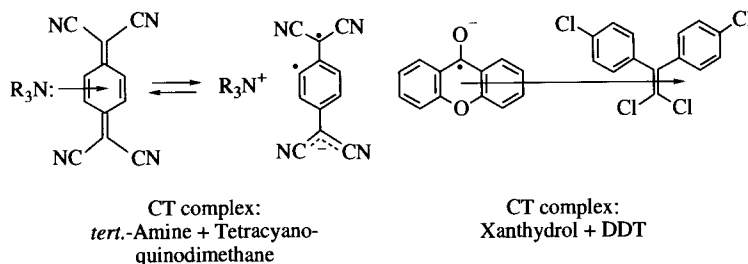
Numerous colorless organic compounds with extended π -electron systems can be converted to colored cations or anions with polymethyne chromophors by protonation or deprotonation. The intense coloration of the corresponding “salts” is usually attributable to the fact that the lone pairs of electrons of the heteroatoms participate in the mesomerism of the conjugated π -electron systems [4].

The well-known BORNTÄGER reaction for the detection of 1,8-dihydroxyanthraquinones is a characteristic example of such halochromism [36–38]:

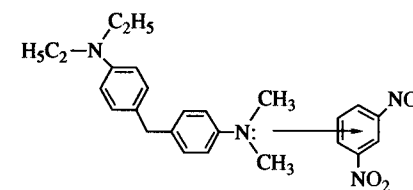


The reaction between 4-nitrophenacyl esters and DMSO/diethylamine, described by KALLMAYER et al., is also a halochromic reaction [39].

Charge transfer complexes (CT complexes) primarily occur in planar organic molecules with conjugated π -electron systems [4]. Examples include:



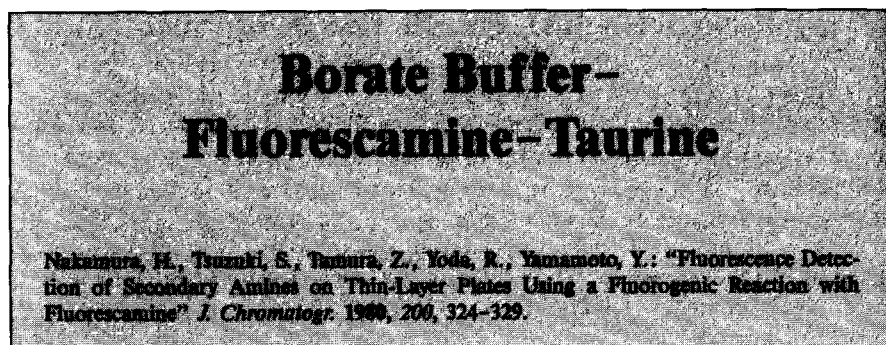
CT complex
Procaine + 1,3,5-Trinitrobenzene



CT complex
4-(Dimethylamino)-4'-(diethylamino)-diphenylmethane
(ARNOLD's reagent) + 1,3-Dinitrobenzene

3.6 Reagent Sequences with Complex Reaction Patterns

In addition to the reagent sequences with clearly detectable reaction mechanism, which have already been described, many sequences of reagents not covered by any of the reaction types described have also found application in thin-layer chromatography. The reaction sequences that remain to be described were all designed to provide as specific a detection of the separated substances as possible.



Reagent Sequence for:

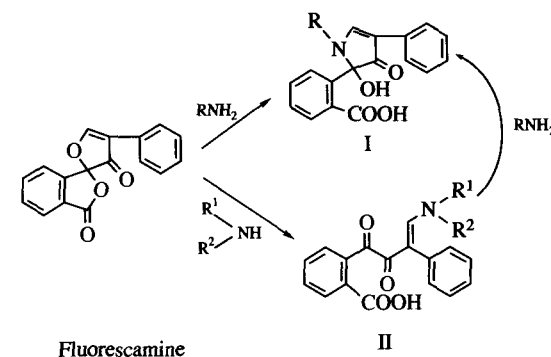
- Secondary amines
e. g. benzimidazole, sarcosine, morpholine

Preparation of the Reagents

- Reagent 1** *Spray solution 1:* 0.05 mol sodium borate buffer (pH 10.5).
- Reagent 2** *Spray solution 2:* Dissolve 20 mg fluorescamine in 100 ml acetone.
- Reagent 3** *Spray solution 3:* 0.2 mol taurine in 0.2 mol sodium phosphate buffer (pH 7.5).

Reaction

Fluorescamine reacts directly with primary amines to yield fluorescent derivatives of the general formula I. On the other hand, secondary amines react in weakly basic medium to yield nonfluorescent derivatives of type II; after the hydrolysis of excess fluorescamine, these are converted to fluorescent products of type I by reaction with a primary amine, e.g. taurine.



Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then heated to 110 °C for 15 min. After cooling to room temperature the layer is sprayed with reagent 2 and then left in the dark at room temperature for 10 min. Finally the chromatogram is sprayed with reagent 3 and heated to 60 °C for 5 min.

Observation under UV light ($\lambda = 254$ or 365 nm) reveals intense fluorescent chromatogram zones on a dark background.

Note: Primary amines yield fluorescent chromatogram zones even before the application of reagent 3. Secondary amines do not yield fluorescent derivatives until they have been treated with reagent 3. Hence, the reagent sequence allows the stepwise detection of primary and secondary amines. Taurine is preferred as the essential component of reagent 3 over the multiplicity of other possibilities because it produces intense fluorescence; it is also not very volatile and is readily available. Amides and substances with peptide linkages, e.g. hippuric acid, are not detected, neither are secondary amines that are volatile at high temperatures.

All traces of mobile phase (and of ammonia in particular) must be removed from the layer, e.g. by heating the chromatogram (10 min 110 °C). The first reagent treatment, including the heat treatment, should be carried out twice if a mobile phase containing acetic acid is used for development.

The detection limits for secondary amines lie between 2 ng (morpholine) and 500 ng (benzimidazole) substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Preparation of the Reagents

- Reagent 1** *Spray solution 1:* Dissolve 2 g 1-chloro-2,4-dinitrobenzene in 100 ml ethanol.
- Reagent 2** *Spray solution 2:* Dissolve 12 g sodium hydroxide pellets in 100 ml methanol.
- Reagent 3** *Ammonia vapor:* Concentrated ammonia solution in the free trough of a twin-trough chamber.

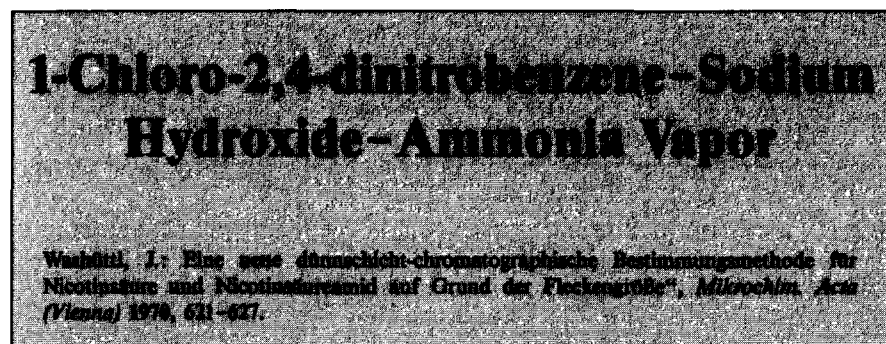
Reaction

Nicotinic acid and related compounds react with 1-chloro-2,4-dinitrobenzene in the manner of the cyanogen bromide reaction to yield derivative I, which possibly also decarboxylates at elevated temperature. In alkaline medium this derivative first adds an hydroxyl ion and then undergoes ring opening to yield the colored derivative II.

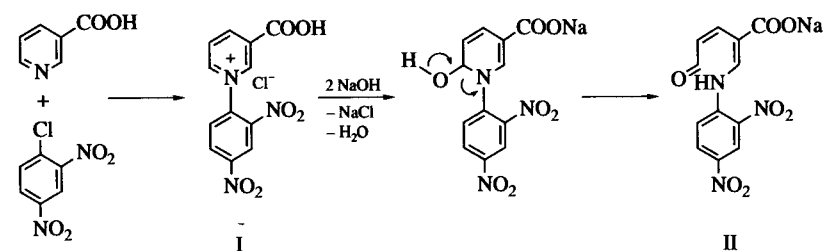
Note: The colors of the chromatogram zones fade relatively quickly. A temperature 180°C should not be exceeded in the first heating step, otherwise the sensitivity detection will be reduced.

The detection limits for nicotinic acid and nicotinamide are 200 ng substance / chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.



Reagent Sequence for:



Method

The chromatograms are dried in a stream of warm air, then sprayed homogeneous with reagent 1 and heated to 180°C for 30 to 45 min. After cooling to room temperature, the chromatograms are sprayed with reagent 2 and dried in a stream of warm air.

Diphenylamine/Iron(III) Chloride/ Sulfuric Acid–Silver Nitrate/ Ammonia

Chawla, H.M., Raihan, N.N., Garg, N.K., Chibber, S.S.: "TLC Separation and Identification of Some Monovalent Anions" *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1980**, *3*, 651–652.

Reagent Sequence for:

- Univalent inorganic anions
e.g. chloride, bromide, iodide, nitrate, thiocyanate

Preparation of the Reagents

- Reagent 1** *Spray solution 1:* Dissolve 1 g iron(III) chloride and 1 g diphenylamine in 100 ml conc. sulfuric acid.
- Reagent 2** *Spray solution 2:* Ammoniacal silver nitrate solution (precise composition not specified).

Reaction

The mechanism of the reaction has not been elucidated. Presumably several reactions occur simultaneously. Thiocyanates react with iron(III) salts with the formation of red-colored complexes. In sulfuric acid medium nitrate or nitrite oxidize diphenylamine to

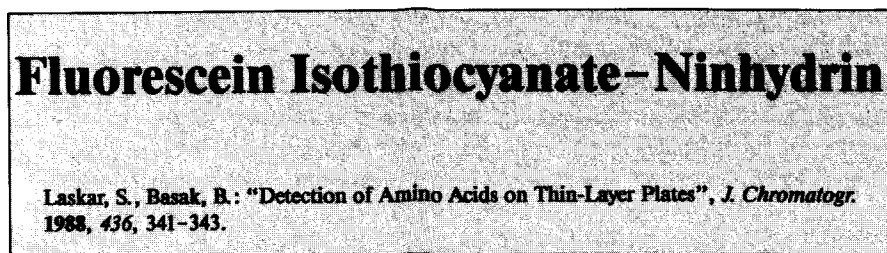
tetraphenylhydrazine, which then undergoes a benzidine rearrangement and the product is oxidized to a blue-colored N,N'-dipenyldiphenyldiphenylhydrazine sulfate. In sulfuric acid medium iodide and bromide are oxidized to the elementary halogens, which are also probably capable of oxidizing diphenylamine to colored derivatives. On the other hand halides are also capable of decomposing the silver tetramine complex (reagent 2) to yield elementary silver, which produces brownish-black chromatogram zones.

Method

The dried chromatograms are sprayed homogeneously with reagent 1 and then with reagent 2 and finally dried in a stream of warm air for 5 to 10 min.

Chromatogram zones of various colors are produced (bromide: yellow, iodide: pale red, thiocyanate: red, nitrate: blue and chloride: black) on a colorless background.

Note: The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.



Reagent Sequence for:

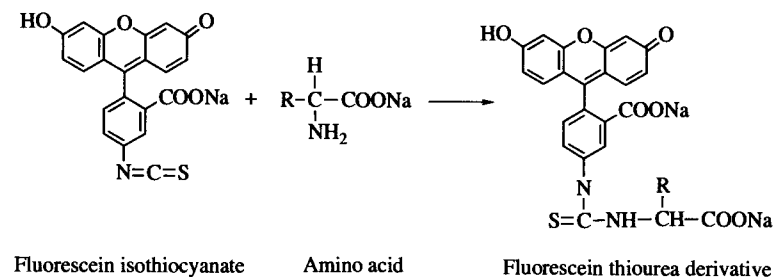
- Amino acids

Preparation of the Reagents

Reagent 1	Formation of thiourea derivatives <i>Spray solution 1:</i> Dissolve 10 mg fluorescein isothiocyanate in 100 ml 0.1 mol sodium hydroxide solution.
Reagent 2	Ninhydrin solution <i>Spray solution 2:</i> Dissolve 250 mg ninhydrin in 100 ml acetone.

Reaction

The amino acids probably react with the fluorescein isothiocyanate to yield fluorescein thiourea derivatives. These are hydrolyzed at elevated temperature in alkaline medium so that the amino groups that are produced can then react with ninhydrin.



Method

The chromatograms dried in a stream of warm air are first sprayed homogeneously with reagent 1 and then heated to 90 °C for 10 min. After cooling to room temperature they are sprayed with reagent 2 and dried in a stream of cold air. Finally they are heated again to 90 °C for 10 min.

The reagent sequence produces colored chromatogram zones of substance-dependent color, some of which appear before the final heating step. The background remains colorless. Some of the zones fluoresce with various colors, when examined under UV light ($\lambda = 280 \text{ nm}$).

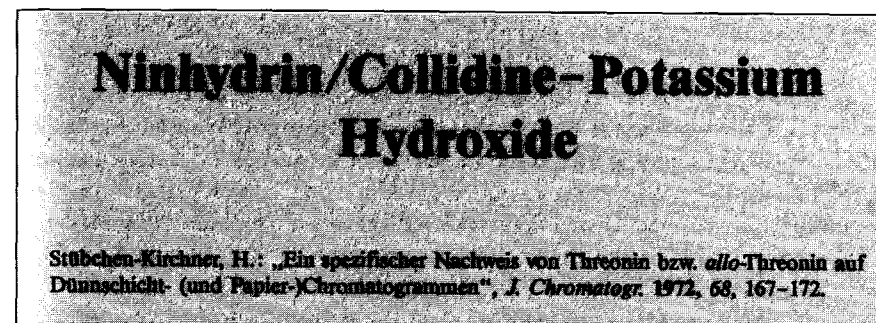
Note: This reagent sequence is less sensitive than ninhydrin alone. However, it possesses the advantage that the colors produced by the individual amino acids vary (Table 1) whereas ninhydrin alone only produces reddish-violet colored zones.

The visual detection limits are 0.3 to 1 μg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Table 1: Color reactions of amino acids with fluorescein isothiocyanate – ninhydrin (extract from original table).

Amino acid	Color of the chromatogram zones in visible light	
	Before heating	After heating
Alanine	grayish-pink	gray
Arginine	mauve	grayish-pink
Asparagine	yellow-orange	orange-yellow
Aspartic acid	pale mauve	dark violet
Cysteine	mauve	brown
Cystine	light beige	yellow
Hydroxyproline	orange-yellow	yellow
Lysine	mauve	grayish-pink
Proline	straw-colored	deep yellow
Serine	pink-violet	grayish-pink



Reagent Sequence for:

- Hydroxyamino acids
e. g. threonine and *allo*-threonine

Preparation of the Reagents

- Reagent 1** **Condensation and decarboxylation**
Spray solution 1: Dissolve 0.4 g ninhydrin and 5 ml 2,4,6-collidine in 2-propanol and make up to 100 ml.
- Reagent 2** **Deprotonation**
Spray solution 2: 1 percent ethanolic potassium hydroxide solution

Reaction

The mechanism of the fluorescence reaction has not been elucidated. See Volume 1 “Ninhydrin – Collidine Reagent” for the ninhydrin reaction.

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until they begin to be transparent and then heated to 80 °C for 20 min. After cooling to room temperature the layers are sprayed several times with reagent 2. After this treatment the chromatograms are stored for an extended period (24 h or longer), under the influence of daylight, in a twin-trough chamber, whose spare trough is filled with 72 percent glycerol (by volume).

Examination under long-wavelength UV light ($\lambda = 365$ nm) reveals pink to brilliant red fluorescent chromatogram zones on a dark background for threonine and *allo*-threonine.

Note: Detection is reported to be specific for threonine and *allo*-threonine. The presence of collidine in reagent 1 is reported to have a favorable effect on the production of the fluorescence. When the substance concentration is high the red fluorescence only occurs at the outer edges of the chromatogram zones (quenching as a result of high concentration). Immediately after treatment with reagent 2 threonine and *allo*-threonine produce characteristic pale green or sometimes yellow green to dirty blue-green fluorescence emissions in long-wavelength UV light ($\lambda = 365$ nm), but these are only visible for 10 to 20 min.

The detection limits for threonine and *allo*-threonine are less than 100 ng substance per chromatogram zone.

The reagent can be employed on cellulose layers.

Potassium Hexaiodoplatinate– Sodium Hydroxide– 1,2-Naphthoquinone-4-Sulfonic Acid

Wallace, J.E., Hamilton, H.E., Skrdlant, H., Burkett, L. Schwertner, H.: "Identification of Selected Antihypertensive Drugs by Thin-Layer Chromatography", *J. Chromatogr.* 1977, 138, 111–118.

Reagent Sequence for:

- Pharmaceuticals and metabolites
 - e.g. diuretics, antihypertensives
 - such as chlorothiazide, hydrochlorothiazide, methyldopa

Preparation of the Reagents

- | | |
|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Reagent 1 | <i>Spray solution 1:</i> Treat 1 ml of a platinum chloride solution (5%) with 1.5 g potassium iodide and mix with 3 ml conc. hydrochloric acid and 11 ml water. |
| Reagent 2 | <i>Spray solution 2:</i> Sodium hydroxide (c = 1 mol/L). |
| Reagent 3 | <i>Spray solution 3:</i> A saturated solution of 1,2-naphthoquinone-sulfonic acid sodium salt in ethanol – water (1+1). |

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are dried in a stream of warm air, then lightly sprayed successively with reagent 1 and reagent 2, followed by reagent 3. Finally they are heated to 110 °C for 5 to 10 min.

Chlorothiazide, hydrochlorothiazide and methyldopa produce pink-colored chromatogram zones on a pale background.

Note: The three reagents should be applied as quickly as possible after each other. In combination with the R_f value, and with UV detection before application of the reagent sequence this procedure allows the identification of therapeutic quantities of thiazide diuretics and methyldopa in urine together with a series of other therapeutic agents. Mobile phase residues e.g. acetic acid, should be completely removed from the chromatograms before application of the reagent sequence.

The detection limits for chlorothiazide, hydrochlorothiazide and methyldopa are 5 µg substance per milliliter urine (working with 5 ml samples).

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sulfuric Acid–Potassium Hexaiodoplatinate

Jukofsky, D., Verebey, K., Mulé, S.J.: "Qualitative Differentiation Between Cocaine, Lidocaine and Cocaine-Lidocaine Mixtures ("Rock Cocaine") Using Thin-Layer Chromatography", *J. Chromatogr.* 1980, 198, 534–535.

Reagent Sequence for:

- Nitrogen-containing compounds
e.g. alkaloids
such as cocaine, lidocaine

Preparation of the Reagents

Reagent 1 *Spray solution 1:* Sulfuric acid (5%).

Reagent 2 *Spray solution 2:* Treat 3 ml hexachloroplatinic(IV) acid solution (10%) with 100 ml aqueous potassium iodide solution (6%) and dilute with 97 ml water.

Reaction

The reaction is a redox reaction whose individual steps have not been elucidated.

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then oversprayed with reagent 2.

Cocaine and lidocaine instantly produce purple-blue chromatogram zones on a colorless background.

Note: The colors of the chromatogram zones change gradually and peripheral rings of color form. The lidocaine zone, for example, becomes brown with a blue-gray ring and the cocaine zone becomes brown with a very weak purple ring, that becomes paler with time. When the cocaine and lidocaine zones are not adequately separated a characteristic blue-gray band-shaped zone appears above the lidocaine and becomes more emphatic as the cocaine zone disappears with time.

The color differences between lidocaine and cocaine zones become clearly apparent after 4 h and are stable for about 24 h. They make it possible to identify cocaine and lidocaine in mixtures, even when the two substances are scarcely separated from each other.

The reagent can be employed on silica gel, kieselguhr, Si 50000, RP and cellulose layers.

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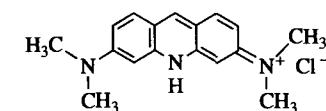
Part II

Reagents in Alphabetical Order

Acridine Orange Reagent

Reagent for:

- Dicarboxylic acids
e. g. succinic acid, glutaric acid [1, 2]
- Aryl- and heteroarylpropionic acids
e. g. carprofen, naproxen [3]



$C_{17}H_{20}ClN_3$

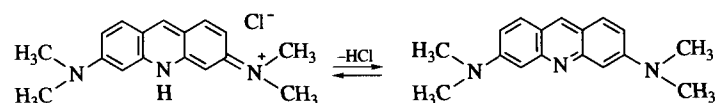
$M_r = 301.82$

Preparation of the Reagent

Dipping solution	Dissolve 0.02 g acridine orange (Basic Orange 14, C.I. 46005) in 100 ml ethanol [3].
Spray solution	Dissolve 0.05 g acridine orange in 100 ml ethanol [1, 2].
Storage	The reagent solutions may be stored for a longer period.
Substances	Acridine orange Ethanol Hydrochloric acid (32%)

Reaction

Acridine orange changes its fluorescence color from pale yellow-green to yellow in a specific pH range (pH 8–10) [1, 4].



Method

The chromatograms are freed from mobile phase (30 min, 120 °C), cooled to room temperature, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution, dried in a stream of cold air for 10 min and then heated to 120 °C for 30 min [1].

Observation under short- and long-wavelength UV light ($\lambda = 254$ nm or 365 nm) reveals yellow to orange fluorescent chromatogram zones on a yellow-green fluorescent background.

Note: Other acridine derivatives can be used as reagents instead of acridine orange (Table 1) [1, 5]. The detection limits lie between a few ng and 100 ng substance per chromatogram zone depending on the substance [1, 3]. If 2-methoxy-9-isothiocyanatoacridine is used as reagent fatty acids can also be detected, whereby the visual detection limits are in the nanogram range at 0.1 μg substance per chromatogram zone [5].

The reagent can, for instance, be employed on silica gel, paraffin-impregnated silica gel, kieselguhr and Si 50000 layers.

Table 1: Fluorescence colors of the chromatogram zones and of the layer background as a function of the reagent employed and of the excitation wavelength [1].

Fluorescence excitation at: Color of:	254 nm		365 nm	
	Background	Zone	Background	Zone
Acridine	pale blue	yellow-green	pale blue	pale yellow
9-Phenylacridine	blue-green	yellow	blue-green	yellow
Acridine orange	yellow-green	red-violet	yellow-green	red
Dibenz[<i>aj</i>]acridine	deep blue	pale blue	deep blue	pale blue
9-Phenoxyacridine	deep blue	pale blue	deep blue	pale blue
9-(<i>p</i> -Methylphenoxy)acridine	deep blue	pale blue	deep blue	pale blue
9-(<i>m</i> -Methylphenoxy)acridine	deep blue	pale blue	deep blue	pale blue
9-(<i>p</i> -Aminophenoxy)acridine	deep blue	pale blue	deep blue	pale blue

Procedure Tested

Carprofen and Naproxen [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation. After sample application the HPTLC plate was preconditioned for 30 min in the mobile phase-free trough of a twin-trough chamber (without filter paper lining).
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Hexane – diethyl ether – 1-butanol – ethyl acetate (65+15+11+9).
Migration distance	6 cm
Running time	20 min

Detection and result: The developed chromatogram was dried for 30 min at 120 °C cooled to room temperature and immersed in the dipping solution for 2 s. Then it was dried in a stream of cold air for 5–10 min.

On visual inspection in daylight carprofen (R_f 40–45) was recognizable as pink-colored chromatogram zone on a yellow background. In long-wavelength UV light ($\lambda = 365$ nm) carprofen appeared as an orange and naproxen (R_f 55–60) as a yellow fluorescent chromatogram zone on a yellow-greenish fluorescent background.

In situ quantitation: For fluorimetric evaluation there was excitation at $\lambda_{\text{exc}} = 313$ nm and the fluorescence emission was measured at $\lambda_{\text{fl}} = 365$ nm (monochromatic filter M 365). This arrangement yielded the most intense signals. (The emission beam at $\lambda = 365$ nm is appreciably more intense than the visible yellow fluorescence.) Further treatment of the chromatogram with liquid paraffin – *n*-hexane (1+2) is not to be recommended.

The photometric detection limit of carprofen was at 4 ng substance per chromatogram zone; 100 ng substance per chromatogram zone could be detected visually.

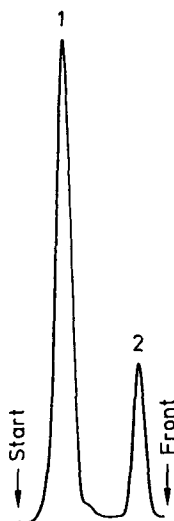


Fig. 1: Fluorescence scan of a chromatogram track with 200 ng each of carprofen (1) and naproxen (2) per chromatogram zone.

References

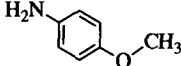
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Ammonium Monovanadate–*p*-Anisidine Reagent

Reagent for:

- Phenols [1–3]

NH_4VO_3
 $M_r = 116.98$
 Ammonium monovanadate

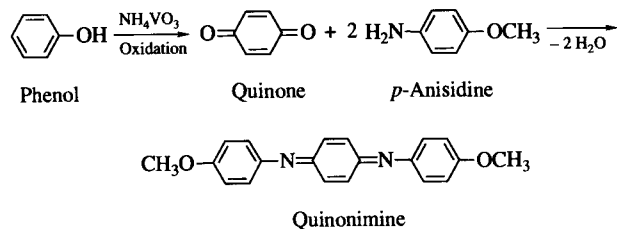

 $\text{C}_7\text{H}_9\text{NO}$
 $M_r = 123.16$
p-Anisidine

Preparation of the Reagent

- | | |
|-------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Solution I | Dissolve 2.5 g ammonium monovanadate (ammonium metavanadate) in 20 ml water and add 20 ml ethanol. Filter off the precipitate that is formed. |
| Solution II | Dissolve 0.5 g <i>p</i> -anisidine in 50 ml ethanol, add 2 ml phosphoric acid (85%) and make up to 100 ml with ethanol. Filter off the precipitate that is formed. |
| Dipping solution | Mix equal volumes of solutions I and II immediately before use. |
| Storage | Reagent solutions I and II may be stored for a longer period. |
| Substances | Ammonium monovanadate
Ethanol absolute
<i>p</i> -Anisidine
Phosphoric acid (85%) |

Reaction

The reaction mechanism has not been elucidated; the ammonium monovanadate presumably oxidizes the phenols to quinones, that then react with *p*-anisidine to form quinonimines.



Method

The chromatogram is dried for 10 min in a stream of warm air and then immersed in the dipping solution for 2 s or sprayed homogeneously with it, dried for 2 min in a stream of warm air and then heated to 110°C for 1 min.

Dimethylphenols yield turquoise-colored chromatogram zones on a violet background, that are converted to blue-violet zones on a flesh-colored background on brief exposure to ammonia vapor.

Note: The detection limits are 20 ng substance per chromatogram zone.

The reagent can, for instance, be employed on silica gel, kieselguhr and on Si 50000 layers.

Procedure Tested

Dimethylphenols [1–3]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK); before application of the samples the layer was developed once to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110°C for 30 min.
Mobile phase	Toluene
Migration distance	6 cm
Running time	10 min

Detection and result: The chromatogram was dried for 10 min in a stream of warm air and immersed in the dipping solution for 2 s, dried for 2 min in a stream of warm air and heated for 1 min to 110°C (Thermoplate DESAGA). 2,5-Dimethylphenol (R_f 25–30) and 2,6-dimethylphenol (R_f 40–45) appeared as turquoise-colored chromatogram zones on a violet background and were converted to blue-violet zones on a flesh-colored background on exposure to ammonia vapor for 5 min.

In situ quantitation: The absorption photometric scan in reflectance was carried out at $\lambda = 590$ nm after exposure to ammonia vapor. The detection limits were 20 ng substance per chromatogram zone (Fig. 1).

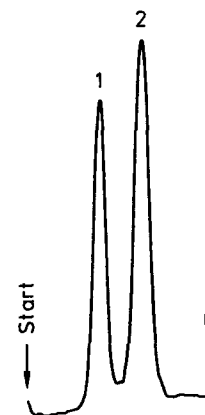


Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone 1 = 2,5-dimethylphenol, 2 = 2,6-dimethylphenol.

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Ammonium Thiocyanate Reagent (Ammonium Rhodanide Reagent)

Reagent for:

● Cations	NH_4SCN
e. g. cobalt(II), iron(III) [1]	$\text{CH}_3\text{N}_2\text{S}$
rhenum(VII), molybdenum(VI), vanadium(V) [2]	$M_r = 76.12$

Preparation of the Reagent

Dipping solution	Dissolve 500 mg ammonium thiocyanate in 45 ml acetone and add 5 ml glacial acetic acid [1].
Storage	The dipping solution may be stored for at least 1 week.
Substances	Ammonium thiocyanate Acetone Acetic acid (100%)

Reaction

Thiocyanate ions form stable complex salts, e. g. intense red with iron(III) and pale blue with cobalt(II) cations.

Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 2 s or homogeneously sprayed with it and then dried for 3 min in a stream of cold air.

Cobalt produces pale blue and iron red-brown chromatogram zones on a colorless background.

Note: Substances that form more stable colorless complexes with the metal ions (e.g. EDTA, phosphates, phosphonic acids etc.) than thiocyanate interfere with the reaction.

Re(VII), Mo(VI) and V(V) cations are detected by first spraying the chromatogram with tin(II) chloride solution (10% in 6 N hydrochloric acid) and then with ammonium thiocyanate solution (50% in water). This leads to the formation of orange, pink or yellow-colored complexes [2].

The detection limits for iron and cobalt cations on cellulose layers are 2 and 20 ng substance per chromatogram zone [1].

The reagent can be used, for example, on aluminium oxide and on cellulose layers.

Procedure Tested

Iron(III) and Cobalt(II) Ions [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Cellulose F _{254s} (MERCK); before application of the samples the layer was developed once to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110°C for 15 min.
Mobile phase	Methanol – hydrochloric acid (32%) – water (80+16+4).
Migration distance	5 cm
Running time	30 min

Detection and result: The developed chromatogram was dried for ca. 5 min in a stream of cold air, immersed in the dipping solution for 2 s and then dried for 3 min in stream of cold air.

Cobalt(II) ions (hR_f 65–70) yielded pale blue and iron(III) ions (hR_f 85–90) red-brown chromatogram zones on a colorless background. The detection limits per chromatogram zone were 2 ng for iron and 20 ng for cobalt.

In situ quantitation: The absorption photometric scan in reflectance was carried out at $\lambda_{\max} = 610$ nm for cobalt and at $\lambda_{\max} = 480$ nm for iron (Figure 1).



Fig. 1: Reflectance scans of a chromatogram track with 200 ng cobalt(II) chloride and 50 ng iron(III) chloride per chromatogram zone. A) scanned at $\lambda = 610$ nm and B) at $\lambda = 480$ nm (different reproduction scales!): 1 = cobalt(II) ions, 2 = iron(III) ions

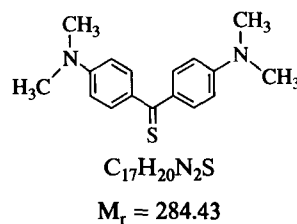
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4,4'-Bis(dimethylamino)- thiobenzophenone Reagent (Michler's Thioketone)

Reagent for:

- Organomercury compounds [1-5]

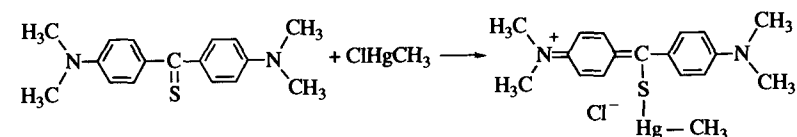


Preparation of the Reagent

Stock solution	Dissolve 1 g 4,4'-bis(dimethylamino)thiobenzophenone (MICHLER's thioketone) in 100 ml chloroform.
Dipping solution	Dilute 1 ml stock solution to 100 ml with chloroform.
Storage	The stock solution is stable for several weeks in the refrigerator. The dipping solution is light-sensitive and should, therefore, always be made up fresh.
Substances	4,4'-Bis(dimethylamino)thiobenzophenone Chloroform

Reaction

The reaction has not been elucidated. Presumably MICHLER's thioketone reacts with organomercury compounds to yield intensely colored, mesomer-stabilized diphenyl-methane derivatives.



Method

The developed chromatogram is dried in a stream of cold air, immersed in the dipping solution for 2 s, then dried in a stream of warm air for 5 min and heated to 110°C for 1-2 min until the coloration reaches its maximum.

Violet chromatogram zones are formed on a pale yellow to pale green background

Note: The detection limits are 1-2 ng substance per chromatogram zone.

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers

Procedure Tested

Organomercury Compounds [1-3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK); before application of the samples the layer was developed once to its upper edge with chloroform - methanol (50 + 50) to precleanse it and then dried at 110°C for 30 min.

Mobile phase *n*-Hexane – diisopropyl ether – tetrahydrofuran (85+10+5).

Migration distance 5 cm

Running time 16 min

Detection and result: The chromatogram was freed from mobile phase for 15 min in a stream of cold air and immersed in the dipping solution for 2 s, dried for 5 min in a stream of warm air and then heated to 110–130 °C for 1–2 min, until the color of the chromatogram zones reached maximum intensity.

Methylmercuric chloride (hR_f 20–25), ethylmercuric chloride (hR_f 25–30), phenylmercuric chloride (hR_f 35–40), dimethylmercury (hR_f 65–70) and diphenylmercury (hR_f 75–80) appeared as violet zones on a pale yellow to pale green background.

Note: With the mobile phase described a pale colored β -front appeared at hR_f 5–10, but it did not affect the interpretation of the chromatogram.

In situ quantitation: The absorption photometric scan in reflectance was carried out at $\lambda = 560$ nm. The detection limits lie between 1 and 2 ng substance per chromatogram zone.

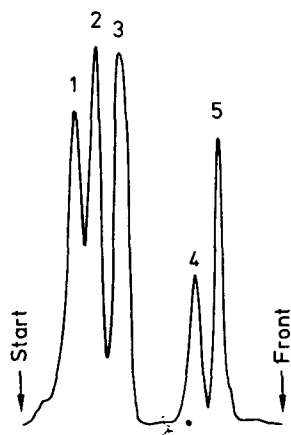


Fig. 1: Reflectance scan of a chromatogram track with 30 ng substance (calculated as Hg) per chromatogram zone: 1 = methylmercuric chloride, 2 = ethylmercuric chloride, 3 = phenylmercuric chloride, 4 = dimethylmercury, 5 = diphenylmercury.

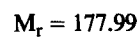
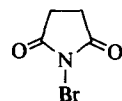
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N-Bromosuccinimide Reagent

Reagent for:

- α -Hydroxyquinones, 5-hydroxyflavones [1]
- Amino acids, Z-protected amino acids
e.g. histidine and histidine derivatives [2, 3]
carnosine [2]

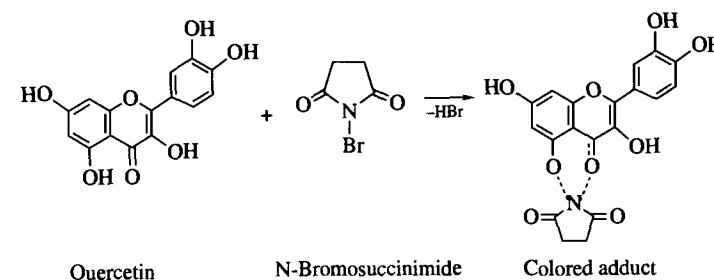


Preparation of the Reagent

Dipping solution	Dissolve 0.5 g N-bromosuccinimide in 100 ml acetone [3].
Spray solution	Dissolve 0.5 g N-bromosuccinimide in 25 ml 1-butanol [1].
Storage	The reagent solutions may be stored for ca. 1 week in the refrigerator [3].
Substances	N-Bromosuccinimide Acetone 1-Butanol

Reaction

The mechanism of the reaction has not yet been elucidated [4]. In the case 5-hydroxyflavonoids it is assumed that colored adducts are formed [1].



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and then heated to 120 °C for 20 min.

This yields yellow to brownish colored chromatogram zones, which emit pale blue fluorescence on a dark background when excited with long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: The detection limits for α -hydroxyquinones and 5-hydroxyflavones are 5 μg and for histidine 20 ng substance per chromatogram zone [3].

The reagent can be used on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Histidine and N- α -Z-L-Histidine [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Chloroform – methanol – ammonia solution (32%) (20+16+10).
Migration distance	7 cm
Running time	30 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air for 15 min, immersed in the dipping solution for 3 s, dried in a stream of cold air for 5 min and then heated to 120 °C for 20 min. Finally, the chromatogram was immersed for 2 s in a solution of liquid paraffin – *n*-hexane (1+2) in order to stabilize and enhance the fluorescence (factor ca. 4).

Histidine (*hR_f* 35–40) and N- α -Z-L-histidine (*hR_f* 45–50) yielded brown chromatogram zones, with a pale blue fluorescence on a dark background under long-wavelength UV light ($\lambda = 365$ nm). The detection limits lay at 20 ng substance per chromatogram zone.

In situ quantitation: Fluorimetric evaluation was carried out with excitation at $\lambda_{\text{exc}} = 365$ nm and the fluorescence emission was measured at $\lambda_{\text{fl}} > 400$ nm (cut off filter K 400).

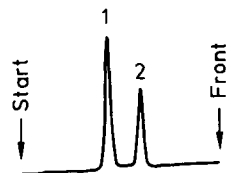


Fig. 1: Fluorescence scan of a chromatogram track with 200 ng each of histidine (1) and N- α -Z-L-histidine (2) per chromatogram zone.

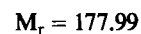
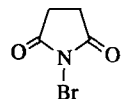
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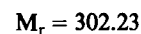
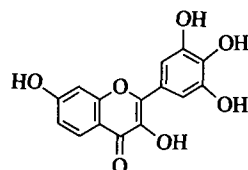
N-Bromosuccinimide–Robinetin Reagent

Reagent for:

- Pesticides
e. g. thiophosphoric acid insecticides [1, 2]



N-Bromosuccinimide



Robinetin

Preparation of the Reagent

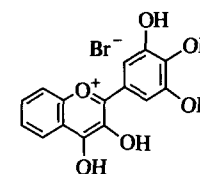
Solution I	Make 3.2 ml bromine up to 100 ml with carbon tetrachloride in a measuring cylinder [1].
Dipping solution I	Dissolve 50 mg N-bromosuccinimide in 50 ml acetone [3].
Dipping solution II	Dissolve 50 mg robinetin in 70 ml ethanol [3].
Storage	The dipping solutions may be stored in the refrigerator at 4 °C for ca. 2 weeks [3].

Substances

N-Bromosuccinimide
Robinetin
Ethanol
Acetone
Bromine
Carbon tetrachloride

Reaction

It is assumed that the hydrogen bromide released on the oxidation of thiophosphoric acid insecticides with N-bromosuccinimide or bromine vapors forms intensely fluorescent salt-like derivatives with 3-hydroxyflavones – such as robinetin [1, 2, 4].



Method

The chromatograms are freed from mobile phase in a stream of warm air and then heated at 105 °C for 5 min, immersed in the dipping solution I for 3 s [3] or placed still warm for 10 s in a twin-trough chamber, whose vacant trough has been filled with 10 ml of solution I [5, 6]. Then after drying in a stream of cold air (after evaporation of the excessive bromine vapor) the chromatograms are immersed in dipping solution II for 3 s or evenly sprayed with it until the layers begin to be transparent and finally heated at 105 °C for 5 min.

In long-wavelength UV light ($\lambda = 365 \text{ nm}$) yellow-green fluorescent chromatogram zones are visible on a weakly fluorescent background.

Note: A range of pesticides can be detected on cellulose layers using 3-hydroxyflavones without prior bromination. Thus, the natural fluorescence of robinetin or fisetin, which is weak in a non-polar environment, is significantly enhanced by the presence of polar pesticides [2, 5, 7, 8].

The detection limits for thiophosphate insecticides are 40–100 ng substance per chromatogram zone [1].

The reagent can be used on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Thiophosphoric Acid Insecticides [3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ , extra thin layer (MERCK), that had been prewashed by immersing overnight in 2-propanol and then dried at 110 °C for 30 min.
Mobile phase	<i>n</i> -Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (543+200+130+127+1).
Migration distance	8 cm
Running time	35 min

Detection and result: The chromatogram was dried in a stream of warm air for 3 min, immersed in dipping solution I for 5 s, dried in a stream of cold air for 3 min and then immersed in dipping solution II for 3 s. It was then heated to 110 °C for 5 min, cooled to room temperature and dipped in a solution of liquid paraffin – *n*-hexane (1+2) for 1 s in order to stabilize and enhance the fluorescence. The chromatogram was then dried in a stream of cold air and evaluated after allowing to stand for ca. 30 min.

In long-wavelength UV light ($\lambda = 365$ nm) oxydemeton-methyl (hR_f 5–10), omethoate (hR_f 15–20), demeton-S-methylsulfon (hR_f 30–35), methamidophos (hR_f 40–45), dimethoate (hR_f 55–60), *trans*-mevinphos (hR_f 60–65), *cis*-mevinphos (hR_f 70–75), dichlorophos (hR_f 75–80) and trichlorfon (hR_f 85–90) appeared as yellow fluorescent chromatogram zones on a weakly fluorescent background.

The detection limits lay at 100 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric evaluation was carried out at $\lambda_{exc} = 365$ nm at the fluorescence emission $\lambda_{fl} > 430$ nm was measured (cut off filter FI 43).

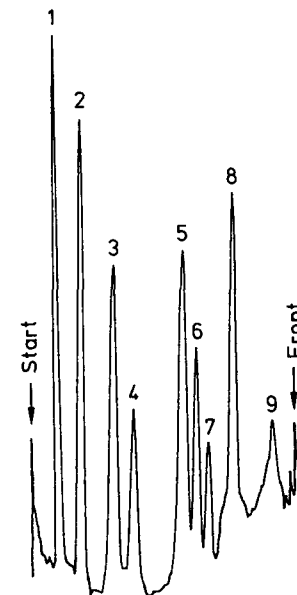


Fig. 1: Fluorescence scan of a chromatogram track with 300 ng of each substance per chromatogram zone:

1 = oxydemeton-methyl, 2 = omethoate, 3 = demeton-S-methylsulfon, 4 = methamidophos, 5 = dimethoate, 6 = *trans*-mevinphos, 7 = *cis*-mevinphos, 8 = dichlorophos, 9 = trichlorfon.

References

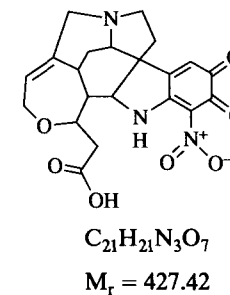
- [1] Frei, R. W., Mallet, V., Pothier, C.: *J. Chromatogr.* **1971**, *59*, 135–140.
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Cacotheline Reagent

Reagent for:

- Ascorbic acid [1, 2]



Preparation of the Reagent

Dipping solution	Dissolve 50 mg cacotheline in 50 ml water and make up to 100 ml with ethanol [2].
Spray solution	Dissolve 1 g cacotheline in 50 ml water [1].
Storage	The reagent solutions should always be made up fresh.
Substances	Cacotheline Ethanol

Reaction

Cacotheline is a redox indicator which is yellow in the oxidized form and reddish-violet in the reduced form.

Method

The chromatograms are dried in a stream of warm air, immersed in the dipping solution for 10 s or sprayed homogeneously with the spray reagent and then dried in a stream of cold air [2] or heated briefly to 110 °C [1].

Ascorbic acid produces reddish-brown to violet chromatogram zones on a yellow background [2].

Note: Dehydroascorbic acid does not react [2, 3].

The detection limit for ascorbic acid is less than 100 ng substance per chromatogram zone [2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Ascorbic Acid [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Ethanol (96%) – acetic acid (10%) (95+5).
Migration distance	5 cm
Running time	30 min

Detection and result: The dried chromatogram was immersed in the dipping solution for 3 s and then heated briefly to 110 °C.

Ascorbic acid (hR_f 50–55) appeared as a brown-red chromatogram zone that was only stable for ca. 20 min; the background was yellow. The detection limit was less than 100 ng substance per chromatogram zone.

Note: Dehydroascorbic acid, the decomposition product of ascorbic acid, does not react. But it can be detected as a yellow-orange chromatogram zone (hR_f 65–70) by further treatment of the chromatogram with 2,4-dinitrophenylhydrazine. This sequence

of reagents, which can also be applied in the reverse order, leads to the disappearance of the red-brown coloration of the ascorbic acid zone within 15 to 20 min!

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 500$ nm (Fig. 1).

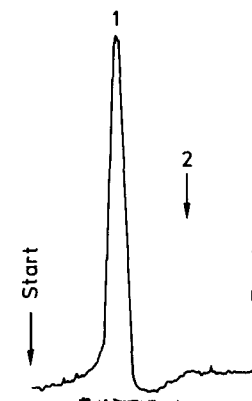


Fig 1: Reflectance scan of a chromatogram track with 500 ng each of ascorbic acid (1) and dehydroascorbic acid (2) per chromatogram zone.

References

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Chloramine T Reagents

A variety of chloramine T reagents, involving the use of chloramine T for chlorination or oxidation reactions, have been described in the literature. These are described in detail individually in the following monographs.

In these reagents chloramine T — a white powder containing approximately 25% active chlorine — is used as a substitute for hypochlorite or chlorine gas. The reactions take place in either acid (hydrochloric acid, sulfuric acid, trichloroacetic acid) or alkaline medium (sodium hydroxide).

Comparative investigations have revealed that varying the acid used or replacing it by a base is generally accompanied by changes — sometimes drastic — in the sensitivity of detection. The information that follows is intended to help the TLC user to choose and optimize the most suitable chloramine T reagent for a particular application:

1. When using chloramine T-mineral acid reagents care should be taken to treat the layer with chloramine T first and then with mineral acid. The sensitivity is considerably reduced if the plate is, for example, merely dipped once in a combined reagent consisting of chloramine T in 5 percent sulfuric acid.
2. Care must be exercised in the choice of acid employed in chloramine T — mineral acid reagent since the detection sensitivity and also the color of the fluorescences produced depend to a significant extent on the choice of acid. This is illustrated for the purine derivatives caffeine, theobromine and theophylline in Figure 1 and Table 1.
3. Only theophylline yields an intensely fluorescent derivative under long-wavelength UV light when treated with chloramine T — sodium hydroxide reagent. The purine derivatives caffeine and theobromine investigated at the same time fluoresce very weakly or not at all.
4. *Digitalis* glycosides that react well with various chloramine T — trichloroacetic acid or mineral acid reagents are not excited to fluorescence after treatment with chloramine T — sodium hydroxide.
5. The phenols pyrocatechol, resorcinol and hydroquinone can be detected with all chloramine T reagents. The detection sensitivity is about the same with chloramine T — sodium hydroxide and chloramine T — trichloroacetic acid. In all cases the detection limits are ca. 75 ng substance per chromatogram zone after the plate has been subsequently dipped in a paraffin oil solution. Somewhat less favorable detection limits of 150 to 200 ng substance per chromatogram zone are obtained after treatment with chloramine T — hydrochloric acid and chloramine T — sulfuric acid.

6. Exposure to hydrochloric acid vapor instead of application of 5% methanolic hydrochloric acid leads to approximately comparable results.

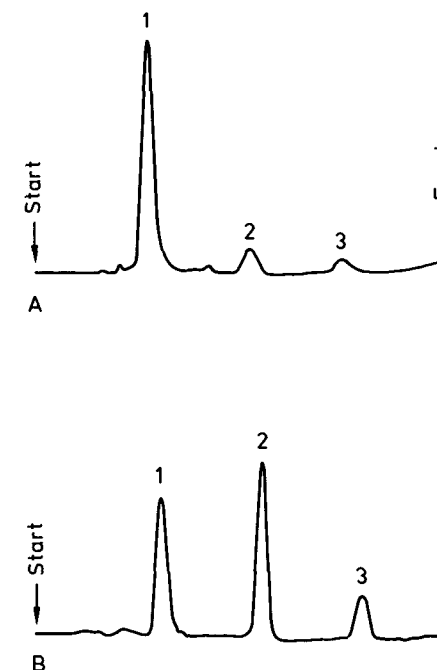


Fig. 1: Comparison of the detection sensitivity after derivatization of three purine derivatives v chloramine T — sulfuric acid (A) and chloramine T — hydrochloric acid (B). Measurements: $\lambda_{\text{exc}} = 365 \text{ nm}$, $\lambda_{\text{em}} = 440 \text{ nm}$ (monochromatic filter M 440): 1 = theophylline, 2 = theobromine, 3 = caffeine.

Table 1: Fluorescence color and detection limits of the derivatives formed by reaction with various reagents incorporating chloramine T.

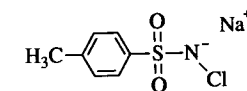
Substance *	HCl	Chloramine T reagent with		
		H ₂ SO ₄	CCl ₃ COOH	NaOH
Theophylline	yellow-green 100	blue 100	blue 100	blue 50
Theobromine	blue 100	yellow 50	yellow 300	no fl. —
Caffeine	blue 200	yellow 300	blue 300	no fl. —
Digoxin	blue 50	blue 50	blue 50	no fl. —
Digitoxin	yellow 50	yellow 50	yellow 50	no fl. —
Pyrocatechol	pale yellow 100	pale yellow 75	yellow-orange 75	yellow 75
Resorcinol	pale yellow 100	pale yellow 100	yellow 75	yellow-orange 75
Hydroquinone	yellow 100	yellow 75	red-brown 75	red-brown 75

* Fluorescence colors produced on excitation at $\lambda = 365$ nm; detection limits in ng per chromatogram zone, after dipping in liquid paraffin – *n*-hexane (1+2).

Chloramine T–Mineral Acid Reagent

Reagent for:

- Purine derivatives [1–3]
e.g. caffeine, theophylline, theobromine
- Steroids, sterols [4, 5]
e.g. dehydroepiandrosterone, cholesterol, solasodine, estriol, testosterone



HCl	H ₂ SO ₄	C ₇ H ₇ ClNNaO ₂ S · 3H ₂ O
M _r = 36.46	M _r = 98.08	M _r = 281.69
Hydrochloric acid	Sulfuric acid	Chloramine T

Preparation of the Reagent

Dipping solution I Dissolve 2.5 g chloramine T in 20 ml water and dilute with 30 ml methanol [6, 7].

Dipping solution II Cautiously mix 47.5 ml methanol with 2.5 ml conc. sulfuric acid and 2.5 ml hydrochloric acid with cooling [6, 7].

Spray solution I Dissolve 10 g chloramine T in 100 ml water [1–3].

Spray solution II Hydrochloric acid (c = 1 mol/L) [1–3].

Spray solution III Dissolve 2 g chloramine T in 100 ml conc. sulfuric acid [4, 5].

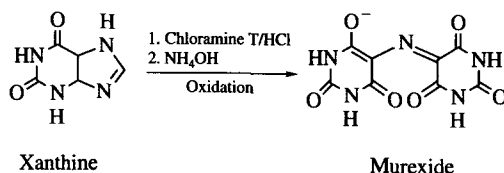
Storage Contrary to what literature reports spray solutions I and II should always be made up fresh; as should dipping solution I. Dipping solution II and spray solution II may be stored for an extended period.

Substances

Chloramine T trihydrate
Hydrochloric acid, 1 mol/L
Hydrochloric acid (32%)
Sulfuric acid (95–97%)
Methanol

Reaction

Purine derivatives (e. g. xanthine) are oxidized by chloramine T in the presence of hydrochloric acid and form purple-red ammonium salts of purpuric acid (murexide) with ammonia. Whether the murexide reaction is also the cause of the fluorescence is open to question.



Method

Purine derivatives: The chromatograms are freed from mobile phase in a stream of warm air, immersed in dipping solution I for 2 s and either placed while still damp in an atmosphere of hydrochloric acid for 10 min [6] or immersed in dipping solution II for 1 s after brief intermediate drying. Alternatively, they can be sprayed homogeneously with spray solution I and then, after brief intermediate drying with spray solution II [1–3]. In both cases the chromatograms are then heated to ca. 100 °C – until the chlorine odor disappears – and then placed for ca. 5 min in the empty trough of a twin-trough chamber which has been filled with ammonia solution (25 %).

Purine derivative yield pinkish-red chromatogram zones on an almost colorless background [1-3]. The chromatogram is then heated to ca. 100°C again until the color development reaches maximum intensity (yellow to orange). On excitation with

long-wavelength UV light ($\lambda = 365$ nm), blue or yellow fluorescent zones become visible now.

Steroids: The developed chromatograms are freed from mobile phase in a stream of cold air, then either immersed for 2 s in dipping solution I and, after brief intermediate drying in a stream of warm air, immersed in dipping solution II for 2 s or homogeneously sprayed with spray solution III and, after being left for 5 min at room temperature, heated at 110°C for 5 min and evaluated.

Even before heating steroids frequently yield characteristic, pale yellow to dark purple colored zones, whose colors generally change on heating [4] and which are usually excited to fluorescence in long-wavelength UV light ($\lambda = 365 \text{ nm}$) [6].

Note: The detection of steroids is more sensitive than with the LIEBERMANN-BURCHARD reaction. Visual detection limits of 0.2 to 5 µg substance per chromatogram zone have been reported [4]; photometric detection limits are appreciably lower being a few nanograms substance per chromatogram zone [6]. The reagent with sulfuric acid is more sensitive than that containing hydrochloric acid for the detection of estrogens [4]. However, the hydrochloric acid-containing chloramine T reagent yields the best coloration results for purine detection; here it is more sensitive than the reagents containing trichloroacetic acid or sulfuric acid [6]. The fluorescence occurring with purines can be increased (factor 2) and stabilized by finally dipping the chromatogram in liquid paraffin — *n*-hexane (1+2) [6].

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers as well as on RP, CN, NH₂ and DIOL phases. Cellulose and polyamide 11 layers change on heating.

Procedure Tested 1

Xanthine Derivatives [6, 7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Ethyl acetate – methanol – ammonia solution (25 (16+4+3)).
Migration distance	6 cm
Running time	15 min

Detection and result: When viewed under short-wavelength UV light ($\lambda = 254$ nm) dark zones were visible due to fluorescence quenching.

With larger amounts of substance red zones appeared on a white background. When viewed under long-wavelength UV light ($\lambda = 365$ nm) clear fluorescence could be seen with lower concentrations if the chromatogram was freed from mobile phase for 5 min in a stream of cold air, immersed in dipping solution I for 2 s and immediately exposed, while still damp, to hydrochloric acid vapors for 10 min (twin-trough chamber, whose second trough had been filled with 10 ml hydrochloric acid (32%)). The chromatogram was heated to 110°C for 10 min and then, after cooling to room temperature, placed in the vacant trough of a twin-trough chamber filled with 10 ml ammonia solution (25%) for 10 min. Then it was heated once again to 110°C for 10 min and, after cooling, dipped in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s in order to increase (factor 2) and stabilize the fluorescence.

In the concentration range above 1 μ g substance per spot, red-colored chromatogram zones (murexide reaction) could be seen on a pale background; these could be excited to blue (caffeine, hR_f 75–80; theobromine, hR_f 55–60) or yellow (theophylline hR_f 35–40) fluorescence on a dark background in long-wavelength UV light ($\lambda = 365$ nm).

If the fluorescence was excited at a wavelength of $\lambda_{exc} = 313$ nm, then the detection limits were 200 ng (caffeine, theophylline) to 400 ng (theobromine) substance per chromatogram zone. At an excitation wavelength $\lambda_{exc} = 365$ nm the detection limits were appreciably lower at 100 ng substance per chromatogram zone for theobromine and theophylline. The sensitivity remained unchanged in the case of caffeine.

In situ quantitation: The fluorimetric evaluation was made either at $\lambda_{exc} = 313$ nm and $\lambda_{fl} > 390$ nm or at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 390$ nm (Fig. 1).

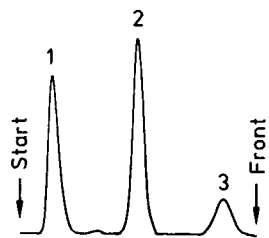


Fig. 1: Fluorescence scan of a chromatogram track with 500 ng each of theophylline (1), theobromine (2) and caffeine (3) per chromatogram zone.

Procedure Tested 2

Estrogens [6]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Toluene – ethanol (90+10).
Migration distance	7 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed in dipping solution I for 2 s and dried in a stream of warm air for 1 min. It was then immersed in dipping solution II for 2 s and heated to 110°C for 10 min. The chromatogram was then placed for 5 min in a twin-trough chamber whose second chamber had been filled with 10 ml ammonia solution (25%), and the heated to 110°C for 5 min. After cooling the chromatogram was immersed for 2 s in liquid paraffin – *n*-hexane in order to increase (factor 2) and stabilize the fluorescence.

After treatment with dipping solution II and subsequent heating the estrogens already appeared as earth brown (estriol, estradiol) and yellow-brown (estrone) chromatogram zones, which could be excited to pale yellow fluorescence on a colorless background in long-wavelength UV light ($\lambda = 365$ nm).

After exposure to ammonia vapor and treatment with paraffin oil estrone ($hR_f = 15$ –20), estradiol (hR_f 30–35) and estrone (hR_f 35–40) appeared as white-yellow fluorescent chromatogram zones on a colorless background, when excited in long-wavelength UV light ($\lambda = 365$ nm). The detection limits for all three estrogens were in the 50 ng range on absorption photometric measurement and 10 ng substance per chromatogram zone on fluorimetric evaluation.

In situ quantitation: The absorption photometric measurement was made at $\lambda = 380$ nm (Fig. 2A) and the fluorimetric evaluation was carried out with excitation at $\lambda_{exc} = 365$ nm and measurement at $\lambda_{fl} > 560$ nm (Fig. 2B).



Fig. 2: Reflectance scan (A) and fluorescence scan (B) of a chromatogram track with 1 μg (A) or 200 ng (B) each substance per chromatogram zone: 1 = estriol, 2 = estradiol, 3 = estrone.

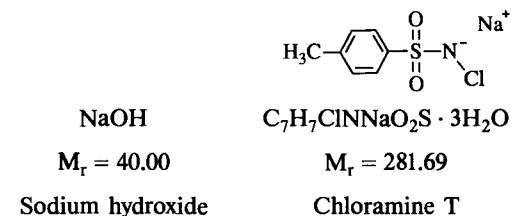
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Chloramine T–Sodium Hydroxide Reagent

Reagent for:

- Polybasic phenols
e. g. 1,2- and 1,3-dihydroxybenzene derivatives,
vicinal trihydroxybenzene derivatives [1]
- Flavonoids [1]
- α -Nitroso- β -naphthol [1]



Preparation of the Reagent

- | | |
|-------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Dipping solution | Dissolve 5 g chloramine T in 50 ml water with shaking. Add 500 mg sodium hydroxide to this solution and dissolve also with shaking. Then dilute with 50 ml methanol. |
| Spray solution | Dissolve 5 g chloramine T in 100 ml 0.5 percent sodium hydroxide solution [1]. |
| Storage | The dipping reagent may be stored in the refrigerator for several weeks. |

Substances Chloramine T trihydrate
Sodium hydroxide pellets
Methanol

Reaction

The mechanism of the reaction has not yet been elucidated; it is assumed that 1,2-dihydroxybenzene is oxidized to quinone and vicinal trihydroxybenzene derivatives to hydroxyquinones [1].

Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution, allowed to stand at room temperature for 5 min and then heated to 120°C for 10 min before examination. 1,2-Dihydroxybenzene derivatives yield yellow zones, 1,3-dihydroxybenzene derivatives purple zones and vicinal trihydroxybenzene derivatives brown chromatogram zones on an almost colorless background [1]. The zones fluoresce under long-wavelength UV light ($\lambda = 365$ nm) [2]; α -resorcinic acid with a peach color, flavonoids and phloroglucinol with a yellowish-red color [1].

Note: Monohydroxybenzene derivatives and vicinal hydroxymethoxybenzene derivatives do not react under these conditions but can be made visible by spraying afterwards with FOLIN-CIOCALTEU's reagent [1]. Flavonoids, anthraquinone derivatives and α -nitroso- β -naphthol yield a red color with alkalis alone [1]. Steroids and aromatic amines do not react [1].

The fluorescence of the chromatogram zones of some phenols can be increased by a factor of 2 and stabilized by dipping the chromatogram in liquid paraffin – *n*-hexane (1+2) [2].

The visual detection limits for polyphenols and flavonoids are 0.2 to 1 μ g substance per chromatogram zone [1]. The photometric detection limits are appreciably lower (see "Procedure Tested").

The reagent can be used on silica gel, kieselguhr, Si 50000, NH_2 and cellulose layers.

Procedure Tested

Dihydroxybenzenes [2, 3]

Method Ascending, one-dimensional development in a trough cham without chamber saturation.

Layer HPTLC plates NH_2 F_{254s} (MERCK).

Mobile phase Toluene – ethyl acetate – ethanol (20+10+10).

Migration distance 6 cm

Running time 15 min

Detection and result: The chromatogram was dried in a stream of cold air for 5 min. Observation under short-wavelength UV light ($\lambda = 254$ nm) revealed dark zones on a pale blue, fluorescent background. Immersion in the dipping solution for 2 s, brief drying in a stream of warm air and then heating to 110–120°C for 10 min yielded, on examination under long-wavelength UV light ($\lambda = 365$ nm) pale yellow (pyrocatechol, hR_f 35–40), yellow (resorcinol, hR_f 45–50) and red-brown (hydroquinone, hR_f 50–55) fluorescent chromatogram zones on a pale blue, fluorescent background. After dipping in liquid paraffin – *n*-hexane (1+2) the detection limits were between 50 and 100 ng substance per chromatogram zone.

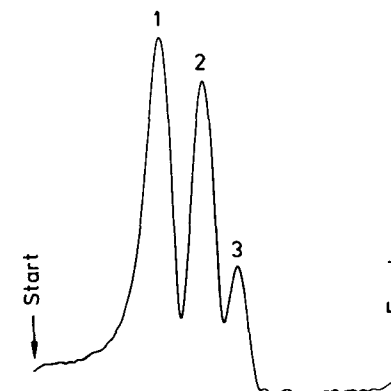


Fig. 1: Fluorescence scan of a chromatogram track with 400 ng each of pyrocatechol (1), resorcinol (2) and hydroquinone (3) per chromatogram zone.

In situ quantitation: Fluorimetric evaluation was carried out under long-wavelength UV light with excitation at $\lambda_{\text{exc}} = 436 \text{ nm}$ and detection at $\lambda_{\text{fl}} > 560 \text{ nm}$ (Fig. 1).

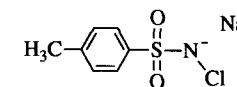
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Chloramine T– Trichloroacetic Acid Reagent (Jensen's Reagent)

Reagent for:

- *Digitalis* glycosides [1–11]
e. g. digoxin, digitoxin, β -methyldigoxin,
 α - and β -acetyldigoxin, gitoxin,
acetyldigitoxin, acetylgitoxin



CCl_3COOH	$\text{C}_7\text{H}_7\text{ClNNaO}_2\text{S} \cdot 3\text{H}_2\text{O}$
$M_r = 163.39$	$M_r = 281.69$
Trichloroacetic acid	Chloramine T

Preparation of the Reagent

Dipping solution	Dissolve 10 g trichloroacetic acid and 0.4 g chloramine T (chloro-4-methylbenzenesulfonamide sodium salt) in a mixture 80 ml chloroform, 18 ml methanol and 2 ml water [11].
Solution I	Dissolve 3 to 5 g chloramine T in 100 ml water.
Solution II	Dissolve 25 g trichloroacetic acid in 100 ml ethanol.

Spray solution	Mix 10 ml solution I with 40 ml solution II immediately before use [1–5]. Other proportions, e. g. 1+15 [6, 10] and 2+8 [7], have also been recommended.
Storage	Solution I should always be made up fresh. The dipping solution may be stored in the refrigerator for several days [11]. Solution II may be stored for longer periods.
Substances	Chloramine T trihydrate Ethanol absolute Trichloroacetic acid Chloroform Methanol

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 to 2 s or homogeneously sprayed with the spray solution and then heated to 100–150°C for 5–30 min [2–7, 10, 11].

Digitalis glycosides yield yellow or blue fluorescent chromatogram zones on a dark background when examined under long-wavelength UV light ($\lambda = 365$ nm) [1, 4, 5–7].

Note: If the mobile phases contains formamide the chromatograms should be freed from it by heating to 130–140°C in the drying cupboard for 1 h before applying the reagent [6].

The detection limits for *digitalis* glycosides are 12–50 ng substance per chromatogram zone [2, 8].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, cellulose and RP layers.

Procedure Tested 1

Cardenolides in Lily of the Valley Extract [12]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK). The layer was prewash before use by developing with chloroform – methanol (50+5) and then drying at 110°C for 30 min.
Mobile phase	Ethyl acetate – methanol – water (81+11+8).
Migration distance	10 cm
Running time	25 min



Fig. 1: Chromatogram of a lily of the valley extract — left before and right after treatment with JENSEN's reagent, photographed in long-wavelength UV light ($\lambda = 365$ nm) [13].

Detection and result: The dried chromatogram was half covered with a glass plate in the direction of development and the other half was homogeneously sprayed with the spray solution. The glass plate was then removed and the whole chromatogram heated to 120°C for 5–10 min, allowed to cool to room temperature and examined under long-wavelength UV light ($\lambda = 365$ nm).

In Fig. 1 it can be seen that the reagent applied to the right-hand side of the chromatogram has, on the one hand, intensified the fluorescent zones of the cardenolides, but that there are, on the other hand, other substance zones whose fluorescence, compared with the natural fluorescence on the left-hand side of the chromatogram, has been weakened appreciably on treatment with the reagent. The reagent is not suitable for in situ quantitation.

Procedure Tested 2

Digitalis Glycosides [14]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ with concentration zone (RIEDEL DE HAEN, MERCK).
Mobile phase	Acetone – dichloromethane (60+40).
Migration distance	5 cm
Running time	8 min

Detection and result: The dried chromatogram was immersed in the dipping solution for 2 s and heated to 110°C for 10 min. Observation in long-wavelength UV light revealed fluorescent zones for digoxin (R_f 30–35) blue and digitoxin (R_f 40–45) yellow.

The fluorescence can be stabilized and enhanced by ca. 20% by dipping in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s. The detection limits after dipping in the paraffin solution are 50 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric evaluation was carried out under long-wavelength UV light at $\lambda_{exc} = 365$ nm and the fluorescence emission was measured at $\lambda_{fl} > 400$ nm (cut off filter K 400) (Fig. 2).

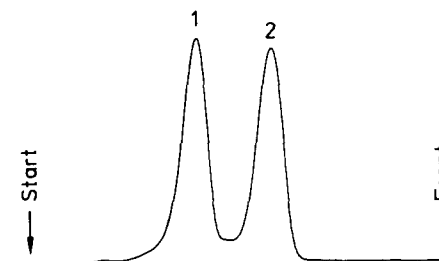


Fig. 2: Fluorescence scan of a chromatogram track with 500 ng each digoxin (1) and digitoxin (2) after treatment of the chromatogram with reagent and paraffin oil solution.

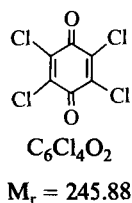
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p-Chloranil Reagent

Reagent for:

- Aromatic amines [1]
e.g. aniline, diphenylamine, anisidine
- Alkaloids [2, 6]
e.g. ephedrine, emetine, cephaeline, cytisine, coniine, γ -coniceine, anabasine, brucine, symphytine, harmaline, berberine, reserpine, papaverine, morphine
- *tert*-N-Ethyl derivatives
e.g. local anesthetics
such as procaine, lidocaine, tolycaïne [7]
- Phenothiazines
e.g. phenothiazine, promazine, promethazine [8]
- Benzodiazepines
e.g. chlordiazepoxide, diazepam, prazepam [2]
- Steroids
e.g. 17 β -estradiol, dienestrol, estrone, stilbestrol, 2 α -xanthatocholestan-3-one [9]
- Antibiotics
e.g. penicillins [10]
such as ampicillin, amoxycillin, cloxacillin, pivampicillin, oxacillin, nafcillin
- Diuretics
e.g. acetazolamide, ethoxolamide, quinethazone, furosemide, benzthiazide, methazolamide [11]
- Antidiabetics [11]
e.g. gliclazide [11]

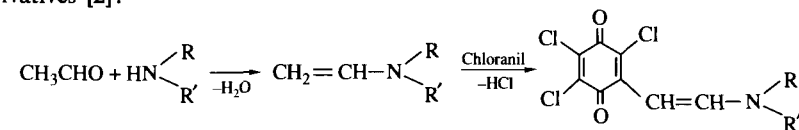


Preparation of the Reagent

Dipping solution	Dissolve 0.5 g <i>p</i> -chloranil in 90 ml ethyl acetate, cautiously mix up to 100 ml with 10 ml sulfuric acid (ca. 96%) and homogenize for 5 min in the ultrasonic bath or by vigorous shaking.
Spray solution I	<i>For alkaloids, phenothiazines, penicillins, diuretics, tert. aliphatic amines and steroids:</i> Dissolve 200 mg to 1 g <i>p</i> -chloranil in 100 ml dioxan, acetonitrile or toluene [2–11].
Spray solution II	<i>For aromatic amines:</i> Dissolve 200 mg <i>p</i> -chloranil in 100 chlorobenzene [1].
Spray solution III	<i>For sec. amines:</i> Dissolve 100 mg <i>p</i> -chloranil in 100 ml dioxan acetonitrile [2].
Storage	The reagent solutions may be stored in the refrigerator for at least one week [2].
Substances	<i>p</i> -Chloranil Ethyl acetate Sulfuric acid (95–97%) Acetonitrile Acetaldehyde Dioxan Toluene Chlorobenzene

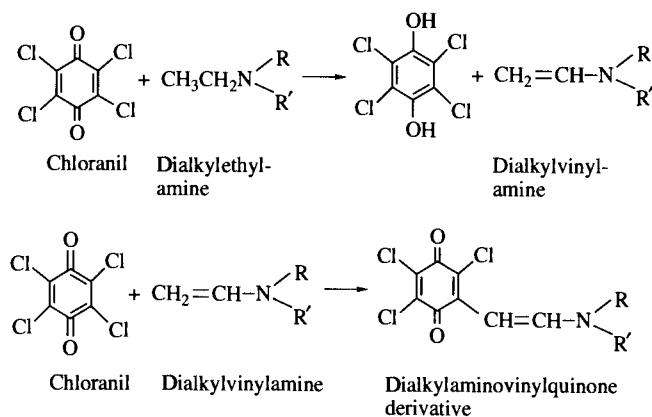
Reaction

Secondary amines react at room temperature with acetaldehyde and *p*-chloranil according to the following scheme to yield intensely blue-colored dialkylaminovinylquinone derivatives [2]:



Dialkylaminovinylquinone derivative

In the case of tertiary N-ethylamine derivatives the N-ethyl group is first selectively oxidized by *p*-chloranil to an enamino group which then condenses with excess *p*-chloranil to a blue aminovinylquinone derivative [7]. Secondary N-ethyl derivatives do not yield blue aminovinylquinone derivatives; they probably react directly with chloranil by nucleophilic attack at one of the four chlorine atoms to yield aminoquinones of other colors [7]. It has also been suggested that some classes of substances react to yield charge transfer complexes [1, 5, 8, 12].



In the case of aromatic amines there is an initial nucleophilic substitution catalyzed by the silanol groups of the silica gel layer to yield arylaminobenzoquinone derivatives, that undergo oxidative cyclization to the corresponding dioxazines [1].

Method

The chromatograms are freed from mobile phase in a stream of warm air, then immersed in the dipping solution for 2 s or homogeneously sprayed with the appropriate spray solution. Then, in the case of N-ethyl derivatives, the plate is heated to 105–110 °C for 2 min to accelerate the reaction [7]. Heating (e.g. to 80–105 °C for 15 min) can also lead to color intensification and color change in the case of other alkaloids [5, 6].

Sec. “amine alkaloids” yield blue-colored chromatogram zones on a colorless to pale yellow background at room temperature [2]. Aromatic amines, phenothiazines, steroid diuretics and penicillins yield yellow, orange or reddish-brown to purple chromatogram zones at room temperature [1, 8–11]. On heating tertiary N-ethyl derivatives also yield blue-colored chromatogram zones, this time on a pale yellow background [7]. Other N-containing pharmaceuticals, e.g. some benzodiazepines, yield grey, brown or violet-brown zones on heating [7].

Note: It is reported that the use of chlorobenzene as solvent is essential when the agent is to be used to detect aromatic amines [1]. In the case of steroids, penicillin diuretics and alkaloids the reaction should be accelerated and intensified by spray afterwards with dimethylsulfoxide (DMSO) or dimethylformamide (DMF), indeed this step makes it possible to detect some substances when this would not otherwise be possible [5, 9–11]; this latter treatment can, like heating, cause color changes [5, 9]. Penicillins and diuretics only exhibit weak reactions if not treated afterwards with DMF [11]. Steroids alone also yield colored derivatives with DMSO [9]. Treatment afterwards with diluted sulfuric acid (c = 2 mol/L) also leads to an improvement in detection sensitivity in the case of a range of alkaloids. In the case of pyrrolizidine alkaloids it is possible to use *o*-chloranil as an alternative detection reagent; however, in this case it is recommended that the plate be treated afterwards with a solution of 2 g 4-(dimethylamino)-benzaldehyde and 2 ml boron trifluoride etherate in 100 ml anhydrous ethanol because otherwise the colors initially produced with *o*-chloranil rapidly fade [12].

The blue derivatives formed with the reagent by alkaloids remain stable for at least one day and usually much longer (cover the chromatogram with a glass plate) [2, 6]. The shade of color produced can be affected by fluorescence indicators incorporated in the silica gel layer [7]. Tertiary amine alkaloids do not react at room temperature with the acetaldehyde-containing reagent [2].

The detection limits (substance per chromatogram zone) are 10 to 20 ng for aromatic amines [1], 100 ng for phenothiazines [8], 0.5 to 2 µg for secondary amine alkaloids, 5 to 50 µg for N-ethyl derivatives [7], 1 to 3 µg for penicillins [10], 1 to 4 µg for diuretics and 1 to 2 µg for a range of steroids [9]. There have been some reports of appreciably lower detection limits of 40–400 ng substance per chromatogram zone and even less for alkaloids [6].

The dipping reagent can be used, for example, on silica gel, kieselguhr, Si 500 RP 18, NH₂, Diol and CN layers. It is not possible to detect aromatic amines on cellulose layers [1].

Procedure Tested

Opium Alkaloids [13]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Acetone – toluene – ethanol – ammonia solution (25%) (40+40+6+2).
Migration distance	7 cm
Running time	7 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s and then heated to 140 °C for 5 min. (Caution! The back of the HPTLC plate is contaminated with sulfuric acid.)

Narceine (hR_f 1–5) yielded red-brown, morphine (hR_f 5–10), codeine (hR_f 15–20) and thebaine (hR_f 35–40) brown-violet, papaverine (hR_f 60–65) light brown and narcotine red chromatogram zones on a colorless background. Since the colors fade in the air it is recommended that the chromatogram be covered with a glass plate.

After treatment with the reagent the detection limits for opium alkaloids are 50–500 ng per chromatogram zone, this is sometimes a somewhat lower sensitivity than that obtained by direct measurement of the UV absorption (cf. Table 1). However, the color reactions provided additional specificity.

Table 1: Comparison of the detection sensitivities

Substance	Detection limits (ng)	
	UV ₂₈₀	<i>p</i> -Chloranil
Narceine	50	100
Morphine	50	50
Codeine	50	50
Thebaine	20	20
Papaverine	5	500
Narcotine	50	50

In situ quantitation: Direct measurement of the UV absorption at wavelength $\lambda = 280$ nm was preferred for quantitative in situ evaluation since the reagent treatment did not yield more exact results.

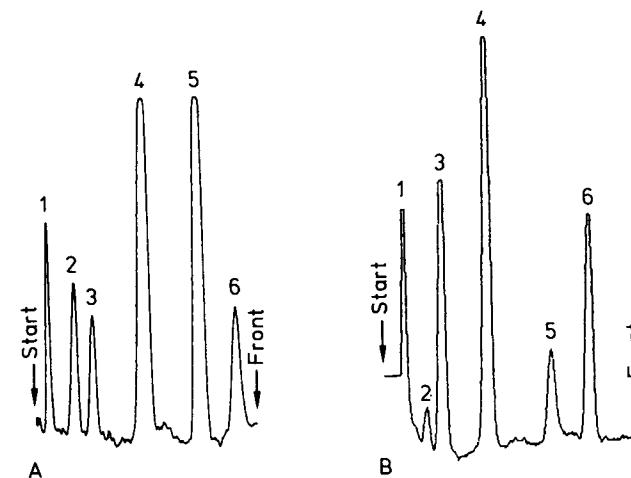


Fig. 1: Reflectance scan of a chromatogram track with 1 µg of each substance per chromatogram zone (exception: papaverine: 5 µg). Chromatogram (A) before and (B) after reagent treatment (registration of B with double sensitivity): 1 = narceine, 2 = morphine, 3 = codeine, 4 = thebaine, 5 = papaverine, 6 = narcotine.

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Chlorine–Potassium Iodide–Starch Reagent

Reagent for:

- Amino, imino, amido groups
 - e.g. peptides [1–4]
 - such as vasotocin analogues [5]
 - e.g. phenylalkylamines
 - such as chlorphentermine, cloforex [6]
 - e.g. parbendazole and its metabolites [7]
 - e.g. phenylbutazone, prenazone [8]
- Phthalimide and derivatives [9]
- Pesticides
 - e.g. fungicides [10]
 - such as benomyl, 2-aminobenzimidazol (2-AB), methyl-2-benzimidazolyl carbamate
 - e.g. triazine herbicides
 - such as simazine, atrazine, propazine
 - prometryn, prometon, ametryn [11]

KI	(C ₆ H ₁₀ O ₅) _n
M _r = 166.01	M _r ≈ 7000
Potassium iodide	Starch

Preparation of the Reagent

Solution I Dissolve 3 g potassium permanganate in 100 ml water.

Solution II Dilute 25 ml hydrochloric acid (32%) with 50 ml water.

Dipping solution Dissolve 250 mg potassium iodide in 25 ml water, mix with solution of 750 mg starch (soluble starch according to ZULKOWSKY) in 25 ml water and dilute with 30 ml ethanol (99.5%) [1]

Spray solution Mix a solution of 0.5–4 g potassium iodide in 50 ml water with a solution of 1.5–2 g starch in 50 ml water [8] and add 20 ml ethanol [9, 10].

Storage The spray solution should always be made up fresh [10].

Substances Potassium iodide
Starch, soluble according to ZULKOWSKY
Potassium permanganate
Hydrochloric acid (32%)
Ethanol

Reaction

Treatment with chlorine gas converts amines to chloramines, whose active chlorine oxidizes iodide to iodine. This then forms the well-known, deep blue iodine-starch complex [13].

Method

The chromatograms are freed from mobile phase in a stream of warm air and treated with chlorine gas for 1–5 min, for example, by placing in the vacant trough of a trough chamber filled with 10 ml each of solution I and solution II [10]. After the excess chlorine has been removed (ca. 5–10 min stream of cold air) the chromatograms are immersed in the dipping solution for 1 s [12] or homogeneously sprayed with spray solution [10].

In general, deep blue chromatogram zones are produced immediately on a color background.

Note: Instead of chlorine gas treatment the chromatograms can be exposed to bromine vapor [8] or sprayed with bleach solution. In the case of phthalimide derivatives chromatograms are heated for 1 h at 180 °C before chlorine treatment [9]. The color

chromatograms remain unchanged for ca. 2 to 3 hours, then they begin to darken [10, 12]. The color of the chromatogram zones turns brown on drying; the original blue color can be regenerated by moistening the chromatogram with water vapor [12].

The starch according to ZULKOWSKY yields a clear solution in cold water without heating; this solution can be diluted with ethanol without precipitation of insolubles. For instance 10 ml of a 3% aqueous starch solution can be mixed with 9 ml ethanol (99.5%) without the precipitation of starch [12].

The detection limits for triazine herbicides are 10–20 ng [11, 12], for phthalimide derivatives 50–100 ng [9] and for fungicides 50–500 ng substance per chromatogram zone [10].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, cellulose, Diol and RP layers [12]. NH_2 phases are not suitable, since the dipping solution elutes a brown-violet solution from the NH_2 layer, which itself remains white [12].

Polyamide and CN phases are not suitable either because the layer background is colored yellow [12].

Procedure Tested

Triazines [12]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene – acetone (85+15).
Migration distance	5 cm
Running time	8 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed for 1 min in a twin-trough chamber in which a chlorine gas atmosphere had previously been generated by pouring ca. 3 ml hydrochloric acid (25%) over ca. 0.2 g potassium permanganate in the vacant trough. The chromatogram was immersed for 1 s in the dipping solution after removal of the excess chlorine (5 min stream of cold air).

The triazines atraton (hR_f 15–20), cyanazin (hR_f 30–35), terbutylazin (hR_f 45–50) and anilazin (hR_f 70–75) immediately appear as violet-blue chromatogram zones on a

colorless background (Fig. 1A). In order to prevent spot diffusion when the chromatogram is dried the TLC plate is placed on a hot plate at 50°C and a stream of warm air was also directed at the layer from a fan (2 min). On drying the color of the chromatogram zones changes from blue-violet to brown (Fig. 1B) and the whole layer becomes dark after a prolonged period. The original blue coloration of the chromatogram zones can be regenerated by moistening the layer with water vapor.

In situ quantitation: The absorption photometric evaluation was carried out in reflectance at wavelength $\lambda = 550 \text{ nm}$ (Fig. 1C).

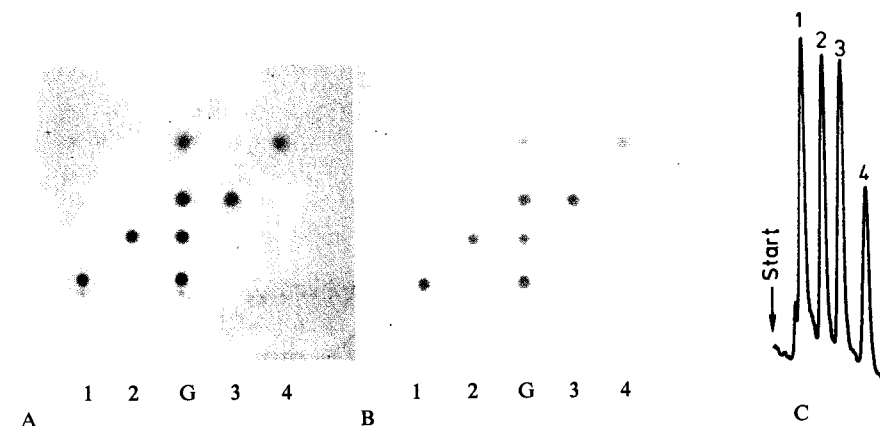


Fig. 1: Chromatogram of triazines (A) after immersion in the dipping solution and (B) after additional heating and (C) reflectance scan of a chromatogram track with 170 ng each of atraton (1), cyanazin (2), terbutylazin (3) and anilazin (4) per chromatogram zone.

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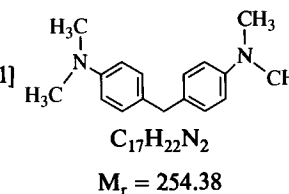
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Chlorine-4,4'-Tetramethyldiamino-diphenylmethane Reagent (TDM Reagent)

Reagent for:

- Amino acids, amides, peptides [1]
- Secondary amines [1, 2]
- Phenols
 - e.g. phenol, *p*-nitrophenol, chromotropic acid
 - N-hydroxysuccinimide, pentachlorophenol [1]
- Triazines
 - e.g. atrazine, trietazine, prometryn, simazine [2]
- Chloranils [3]



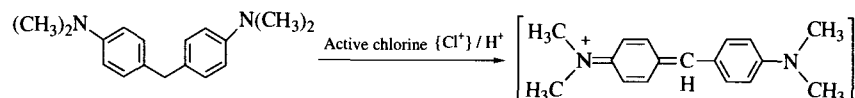
Preparation of the Reagent

- | | |
|---------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Solution I | Dilute 20 ml sodium hypochlorite solution (13–14% active chlorine) with 100 ml water [1]. |
| Solution II | Dissolve 2.5 g 4,4'-tetramethyldiaminodiphenylmethane (TDM, MICHLER's base, N,N,N',N'-tetramethyl-4,4'-diaminodiphenylmethane) in 10 ml glacial acetic acid (yields a greenish solution) and make up to 100 ml with 80% 2-propanol. |
| Solution III | Dissolve 5 g potassium iodide in 100 ml 80% 2-propanol. |
| Solution IV | Dissolve 300 mg ninhydrin in 10 ml glacial acetic acid and dilute to 100 ml with 80% 2-propanol. |

Dipping solution	Mix solutions II and III together and add 1.5 ml solution IV.
Storage	Solutions I to IV may be stored in the refrigerator for several weeks. The dipping solution may be kept in the dark for 1 month at room temperature [1].
Substances	Sodium hypochlorite solution 4,4'-Tetramethyldiaminodiphenylmethane Potassium iodide Ninhydrin Acetic acid (100%) 2-Propanol

Reaction

Presumably the active chlorine of the chloramines formed by reaction with chlorine gas or hypochlorite reacts with TDM in the presence of acetic acid to yield dark blue, mesomerically stabilized quinoid reaction products that possibly rearrange to yield triphenylmethane dyestuffs.



Phenols are probably initially oxidized to quinones, which then presumably react further to yield triphenylmethane dyestuffs.

Method

The chromatograms are freed from mobile phase (15 min 100°C), placed in the empty chamber of a twin-trough chamber containing 20 ml solution I (chlorine chamber) for 1 min or homogeneously sprayed with solution I until the layer begins to be transparent. They are then freed from excess chlorine in a stream of warm air for 30 min and immersed in the dipping solution for 3 s or sprayed homogeneously with it.

Yellow chromatogram zones are first formed at room temperature; the color of these zones generally changes to green and then to blue-black. This color change can be accelerated by heating or irradiating with long-wavelength UV light ($\lambda = 365 \text{ nm}$) [1]; at the same time the background also takes on a greenish color.

Note: The TDM reagent can be used everywhere, where *o*-tolidine is employed. It can also be used on chromatograms, that have already been treated with ninhydrin, PAULY or ammonia perchlorate reagent or with iodine vapor [1]. Water may be used in place of 80% 2-propanol when making up solutions II, III and IV. The chlorine gas atmosphere in the chromatography chamber can also be created by pouring 5 ml hydrochloric acid (ca. 20%) onto 0.5 g potassium permanganate in a beaker; such a chlorine chamber is ready for use after 2 min.

Chloranils, which are formed from polychlorine phenols by heating briefly with conc. nitric acid, can be detected, without chlorine treatment, with TDM reagent, followed by heating (10 min 110°C) [3]. Phenols yield variously colored chromatogram zones (e.g. phenol: mauve, chromotropic acid: grey, 8-hydroxyquinoline: light brown, 4-*tert*-butyl-pyrocatechol: red [1]).

Before treatment with chlorine gas or hypochlorite solution, pyridine-containing mobile phases must be removed completely from the chromatograms, if necessary, by prolonged drying in a stream of warm air [1]. The colored chromatograms may be stored for several days in the dark under cool conditions [1].

In the case of protected peptides, it is necessary to remove the protecting groups by spraying the chromatograms with conc. hydrochloric acid and then heating (20 min, 110°C), before applying the reagent [1].

The detection limits for triazines are 20 ng [2] and for amino acids – with a few exceptions – 0.1 to 1 μg substance per chromatogram zone [1]. In the case of peptides 50 ng can be detected visually.

The reagent can be used on silica gel, kieselguhr, Si 50000, aluminium oxide and cellulose layers.

Warning: 4,4'-Tetramethyldiaminodiphenylmethane is thought to be carcinogenic [4]. Therefore, the dipping solution should be used if possible (gloves!). It is only in this way that it is possible to guarantee that the spray vapors do not come into contact with the skin or respiratory tract.

Procedure Tested

Triazines [5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (RIEDEL DE HAEN)
Mobile phase	Cyclohexane – dichloromethane – dioxan – tetrahydrofuran (80+10+5+5).
Migration distance	7 cm
Running time	20–25 min

Detection and result: The chromatogram was freed from mobile phase and placed in an atmosphere of chlorine gas (twin-trough chamber, containing 20 ml solution I in the second chamber) for 1 min. Then the excess chlorine was removed (30 min stream of warm air), the treated chromatogram immersed in the dipping solution for 3 s and dried on a hotplate (60–70 °C).

Depending on the duration of heating yellow-green to dark green chromatogram zones were produced on a weakly colored background. The detection limits of the triazine herbicides cyanazine (hR_f 25–30), simazine (hR_f 30–35), atrazine (hR_f 40–45), terbutylazine (hR_f 45–50) and anilazine (hR_f 60–65) were 20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at $\lambda = 353$ nm (Fig. 1).

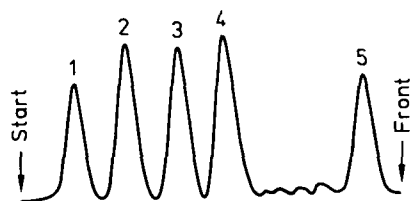


Fig. 1: Reflectance scan of a chromatogram track with 100 ng each of 1 = cyanazine, 2 = simazine, 3 = atrazine, 4 = terbutylazine, 5 = anilazine per chromatogram zone.

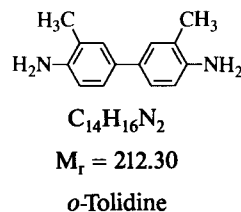
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Chlorine-*o*-Tolidine-Potassium Iodide Reagent (Reindel-Hoppe Reagent)

Reagent for:

- Compounds that can be converted to chloramines [1-4]
e.g. amino acids, peptides, proteins and derivatives [5-13]
such as cyclochlorotin, simatoxin [14]
aspartic acid derivatives [15]
carbobenzoxyamino acids [16]
caprolactam oligomers [17]
phenyl carbamate herbicides
such as chloropropham [18, 19]
urea and urea derivatives [20, 21]
carbamide derivatives [10]
- Pesticides
- Triazines and their hydroxy derivatives [22, 23]
e.g. atrazine, simazine [18, 19, 24]
metribuzine [25]
prometryn [18, 24, 26, 27]
- Vitamins [28-30]
e.g. vitamins B₁, B₂, B₆, nicotinamide, panthenol
- Pharmaceuticals
e.g. sulfonylurea derivatives [31, 32]
meprobamate, barbiturates [33]
sulfonamides [34]
central stimulants [35]

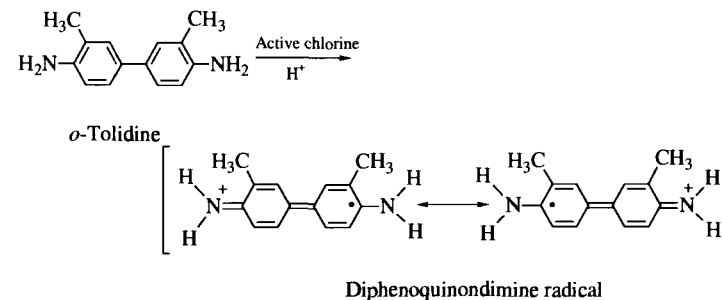


Preparation of the Reagent

Solution I	Dissolve 0.5 g <i>o</i> -tolidine (3,3'- dimethylbenzidine) in 5 ml acetic acid (100%) and make up to 250 ml with water or 80% 2-propanol [26, 27].
Solution II	Dissolve 2 g potassium iodide in 10 ml water.
Dipping solution	Combine solutions I and II and make up to 500 ml with water or 80% 2-propanol [26, 27].
Spray solution	Dissolve 160 mg <i>o</i> -tolidine in 30 ml glacial acetic acid and make up to 500 ml with water; dissolve 1 g potassium iodide in this solution [4, 14, 16, 17, 28-30].
Storage	Dipping and spray solutions may be stored in the refrigerator for 1 week [24, 27].
Substances	<i>o</i> -Tolidine Potassium iodide Potassium permanganate Hydrochloric acid (32%) Acetic acid (100%) 2-Propanol

Reaction

The action of chlorine gas produces, for example, chloramine derivatives from herbicides, amino acids, peptides and proteins; the active chlorine of these derivatives the



oxidizes *o*-tolidine in the presence of acetic acid and potassium iodide to produce a deep blue dyestuff (diphenquinonediimine radical) [1], that exhibits semiquinonoid character [2]. The active chlorine possibly also reacts with the potassium iodide to liberate iodine which is capable of complex formation with the semiquinonoid reaction product, thus deepening its color.

Method

The chromatogram is freed from mobile phase in a stream of cold air and then exposed to an atmosphere of chlorine gas for 30 s – 1 h. This chlorine gas can be generated in a trough chamber by pouring 5 ml ca. 20% hydrochloric acid onto 0.5 g potassium permanganate placed in a beaker; the chlorine gas chamber is ready for use after ca. 2 minutes.

The chromatogram is then completely freed from excess chlorine in a stream of warm air for 30 min, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and dried for 5 min in a stream of warm air [26].

Usually blue-grey colored chromatogram zones are produced on a colorless [19, 24, 25] to pale grey background [26]; cyclochlorotin and simatoxin yield brilliant yellow zones [14] and urea derivatives yellow, green or blue colors [21].

Note: The dipping solution can also be used as a spray reagent. The quantitative scan should not be delayed for more than 1 h since the background begins to darken after this [26]. The chromatogram should not be completely freed from water before exposure to chlorine gas [2], moistening in water vapor has even been suggested [1, 16]. The chromatogram should first only be sprayed or dipped in the reagent at one corner. If the background becomes blue this is a sign that traces of chlorine gas are still present; in such cases the chromatogram should be exposed to air for longer after treatment with chlorine gas [16, 24, 28–30].

Instead of exposing the chromatogram to chlorine gas it can be dipped in a solution of chlorine in carbon tetrachloride [24] or, in some instances, pretreated with sodium hypochlorite [4] or *tert*-butyl hypochlorite solution [8]. Treatment with chlorine gas is not necessary for chloramines; here the chromatogram can be treated with the spray solution immediately after development and drying [36]. A modified reagent includes sodium tungstate solution in making up the spray solution [20]. In some cases the colored zones fade within a few minutes [24]. An additional treatment with 1% ammonium molybdate in acetic acid ($c = 1 \text{ mol/L}$) stabilizes the colored zones [1]. A series of chlorinated pesticides and biphenylenes can be detected with *o*-tolidine followed by exposure to light (see *o*-tolidine – UV light reagent).

The detection limit is 1 ppm for cyclochlorotin and 12–100 ng substance per chromatogram zone for triazines [18, 19, 25–27].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000 and cellulose layers.

Caution: *o*-Tolidine is very toxic and possibly carcinogenic! For this reason the dipping method should be preferred. This is the only way to ensure that the spray mist does not reach the respiratory tract or skin.

Procedure Tested

Triazines [26, 27]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK, RIEDEL-DE-HAEN); before application of the samples the layer is immersed in 2-propanol for 12 hours (preferably overnight) for purification purposes and then activated on a hot plate for 60 min at 110°C.
Mobile phase	1. n-Pentane – chloroform – acetonitrile (50+40+10) (Fig. 1). 2. Cyclohexane – dichloromethane – dioxan – tetrahydrofuran (80+10+5+5) (Fig. 2).
Migration distance	5–7 cm
Running time	20 – 25 min

Detection and result: The chromatogram was freed from mobile phase for 5 min in a stream of cold air and then for 15 min on a hot plate (Thermoplate DESAGA) at 60°C. It was then exposed for 30 s to an atmosphere of chlorine gas, that had been generated in a twin trough chamber by pouring 5 ml hydrochloric acid (ca. 20%) onto 0.5 g potassium permanganate placed in one of the troughs (waiting time 2–5 min after the acid had been poured onto the potassium permanganate). The chromatogram was then freed from excess chlorine for 30 min in a stream of warm air, immersed in the dipping solution for 3 s and dried for 5 min in a stream of warm air.

The substances aziprotryn (hR_f 80–85), dipropetryn (hR_f 70–75), prometryn (hR_f 65–70), ametryn (hR_f 55–60), desmetryn (hR_f 40–45) and methoprotryn (hR_f 30–35) separated using mobile phase 1 and the components terbutylazine (hR_f 45–50), atrazine (hR_f 35–40), simazine (hR_f 30–35) and cyanazine (hR_f 20–25) chromato

graphed with mobile phase 2 all yielded blue-grey chromatogram zones on a pale grey background. Anilazine (hR_f 60–65, mob. ph. 1) did not produce a color, but it could be quantified well without reagent treatment at $\lambda = 223$ nm.

Note: Since the plate background begins to darken after 1 h it is necessary to carry out quantitation within this time.

The detection limits for triazines are in the range 10–20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric measurement in reflectance was carried out at a mean wavelength of $\lambda = 650$ nm (Fig. 1) and 495 nm (Fig. 2).

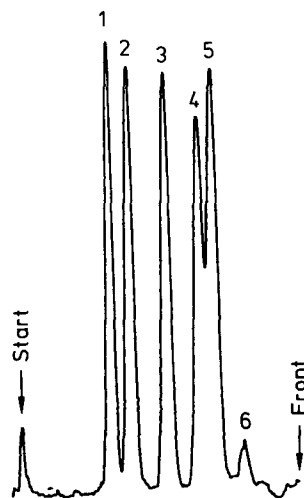


Fig. 1

Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone: 1 = methoprotryn, 2 = desmetryn, 3 = ametryn, 4 = prometryn, 5 = dipropetryn, 6 = aziprotryn

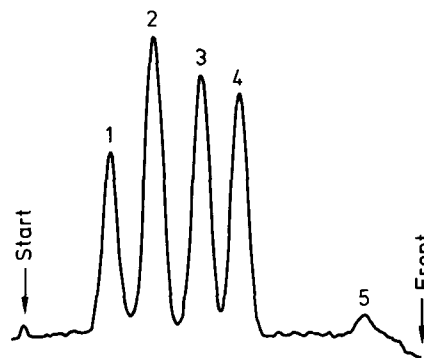


Fig. 2

Fig. 2: Reflectance scan of a chromatogram track with 100 ng substance per chromatogram zone: 1 = cyanazine, 2 = simazine, 3 = atrazine, 4 = terbutylazine, 5 = anilazine

References

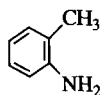
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Chlorine-*o*-Toluidine Reagent

Reagent for:

- Amines, amides
e. g. hexamine, urea derivatives, melamine resins [1, 2]
peptides [3–6]
- Triazines [7]
- Vitamin B₁, B₂, B₆



C_7H_9N
 $M_r = 107.16$

Preparation of the Reagent

Dipping solution	Dissolve 2.5 ml <i>o</i> -toluidine in a mixture of 45 ml diethyl ether and 5 ml glacial acetic acid [7].
Spray solution	Dissolve 5 g <i>o</i> -toluidine in 100 ml glacial acetic acid [1, 2].
Storage	The reagent solutions may be stored for several weeks [1].
Substances	<i>o</i> -Toluidine Acetic acid (100%) Potassium permanganate Hydrochloric acid (25%) Diethyl ether

Reaction

Peptides, for example, yield chloramine derivatives on exposure to chlorine gas; the derivatives oxidize *o*-toluidine to a blue semiquinonoid dyestuff in the presence of acetic acid.

Method

The chromatograms are freed from mobile phase and then treated for 5–10 min with chlorine gas. This can be produced in the vacant trough of a twin-trough chamber by pouring 5 ml hydrochloric acid (ca. 20%) over 0.5 g potassium permanganate. After the excess chlorine has been removed the TLC plates are immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and laid out in the air.

Yellow, green, gray or blue chromatogram zones are produced on a colorless background [1, 2, 7].

Note: Chloramines do not require exposure to chlorine gas before application of *o*-toluidine. A range of halogen-containing substances (e. g. bromazine, hexachlorocyclohexane isomers) can be detected with *o*-toluidine (1% in ethanol) after subsequent irradiation with UV light ($\lambda = 254$ or 366 nm; 10–15 min) [1, 8].

The detection limits for triazines are 300 ng [7] and for urea formaldehyde reaction products they are 1 to 5 μ g substance per chromatogram zone [1].

The reagent can be used on silica gel, kieselguhr and Si 50000 layers. RP, CN, Diol, NH₂ and cellulose layers are not suitable. Amino layers, for example, turn yellow under influence of the reagent [7].

Warning: *o*-Toluidine is highly poisonous and possibly carcinogenic! Therefore, the dipping solution should be employed if possible. This is the only way to ensure that spray vapor is kept away from the respiratory tract or skin.

ing solution and left in the air for a few minutes.

Cyanazin (hR_f 5–10) appeared as gray, terbutylazin (hR_f 20–25) as violet and anilazin (hR_f 35–40) as pale blue chromatogram zones (Fig. 1). The intensity of the spots increased during one hour but did not change thereafter. The detection limits for all three substances were 300 ng per chromatogram zone. These amounts could also readily be detected visually.

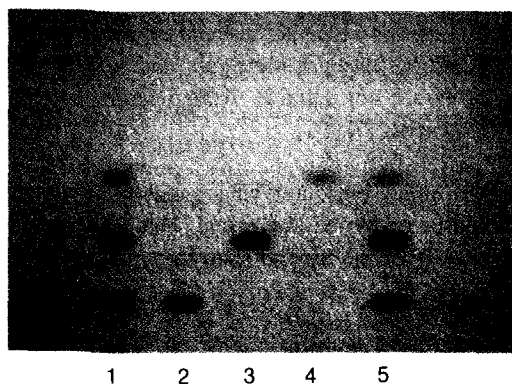


Fig. 1: Thin-layer chromatogram of triazines (amount applied: 4 μ g each substance per chromatogram zone). Tracks 1 and 5 = mixture, Track 2 = cyanazin, Track 3 = terbutylazin, Track 4 = anilazin.

and amiazin (5) per chromatogram zone.

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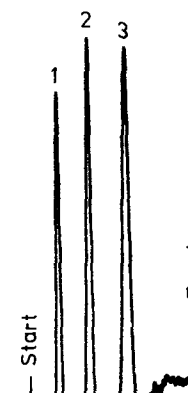
Procedure Tested

Triazines [7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Cyclohexane – dichloromethane – tetrahydrofuran – dioxane (80+10+5+5).
Migration distance	7 cm
Running time	14 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed for 5 min in a twin-trough chamber in which a chlorine gas at-

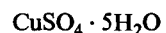
In situ quantitation: Absorption photometric evaluation in reflectance was carried out at wavelength $\lambda = 650$ nm (Fig. 2).



Copper(II) Sulfate– Sodium Citrate Reagent (Benedict's Reagent)

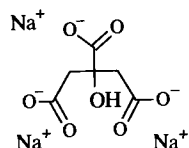
Reagent for:

- Reducing sugars [1]
- Flavonoids [2–5]
- Cumarins [4]



$$M_r = 249.68$$

Copper sulfate



$$M_r = 294.10$$

Sodium citrate

Preparation of the Reagent

Solution I	Dissolve 17.3 g copper(II) sulfate pentahydrate in 100 ml water.
Solution II	Dissolve 173 g <i>tri</i> -sodium citrate dihydrate and 270 g sodium carbonate decahydrate in 600 ml water.
Dipping solution	Slowly add solution I to solution II with stirring and make up to 1 L with water [1].
Storage	The dipping solution can be stored for several weeks at room temperature.
Substances	Copper(II) sulfate pentahydrate <i>tri</i> -Sodium citrate dihydrate Sodium carbonate decahydrate

Reaction

Reducing sugars convert copper(II) salts to red copper(I) oxide. Evidently the phenolic OH groups of many flavonoids and cumarins are also capable of reducing copper(II) probably leading to strongly fluorescent quinoid systems. Substances with *ortho* phenolic OH-groups have their natural fluorescence reduced, those with isolated OH groups have this enhanced [6].

Method

After development the chromatogram is freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 s or homogeneously sprayed with it, dried for 5 min in a stream of cold air and, in the case of flavonoids and cumarins, is immediately examined under long-wavelength UV light ($\lambda = 365 \text{ nm}$) [7]. Reducing sugars are detected by heating to 105°C for 30 min after dipping or spraying [1].

When examined under long-wavelength UV light ($\lambda = 365 \text{ nm}$) cumarins yield light blue [7] and flavonoids yellow-green [2, 3] fluorescent chromatogram zones on a dark background. Reducing sugars yield brilliant orange-colored zones on a colorless to pale beige background.

Note: The dipping solution can also be used as a spray solution [7]. Chromatogram of natural product extracts should always be examined under UV light before using BENEDICT's reagent, since some natural fluorescences are reduced to a greater or lesser degree by the reagent.

The detection limits for cumarins are 5 ng substance per chromatogram zone [7]. They can be appreciably lowered by dipping the mobile phase-free chromatogram in solution of liquid paraffin – *n*-hexane (1+9) [8].

The reagent can be used on silica gel, kieselguhr, Si 50000, cellulose and polyamide layers.

Procedure Tested

Cumarins in Plant Extracts [7, 8]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK); before application of the samples the layer was developed with methanol to precleanse it and then dried at 110 °C for 30 min.
Mobile phase	Acetone – water – ammonia solution (25%) (90+7+3).
Migration distance	7.5 cm
Running time	17 min

Detection and result: The chromatogram was dried in a stream of warm air. Blood-red fluorescent chlorophyll zones were visible in the region of the solvent front. In the case of *Orthosiphon* leaf extract there was an intense pale blue fluorescent sinensetin zone (hR_f 90–95) immediately below this, followed by a series of usually weaker blue fluorescent zones extending right down to the start zone (Fig. 1A).

After application of BENEDICT's reagent (dipping time: 3.5 s; 5 min drying in a stream of warm air) the fluorescence intensity of many of the chromatogram zones is appreciably reduced. At the same time the fluorescence of other zones is increased (Fig. 2), so that in stinging nettle extract, for instance, the scopoletin zone (hR_f 48–53) fluoresced most strongly (Figure 1B). Dipping for 3 s in liquid paraffin – *n*-hexane (1+9) followed by drying in a stream of cold air caused the fluorescence intensity to increase by a factor of 2.

Note: Allowing the sprayed chromatograms to stand for a longer time and, in particular, exposing them to heat, reduces the intensity of the fluorescence of the chromatogram zones.

In situ quantitation: The fading of the fluorescence on exposure to heat and on allowing the chromatograms to stand makes this reagent unsuitable for in situ quantitation. Dipping the chromatograms in paraffin solution does not improve this (Fig. 2).

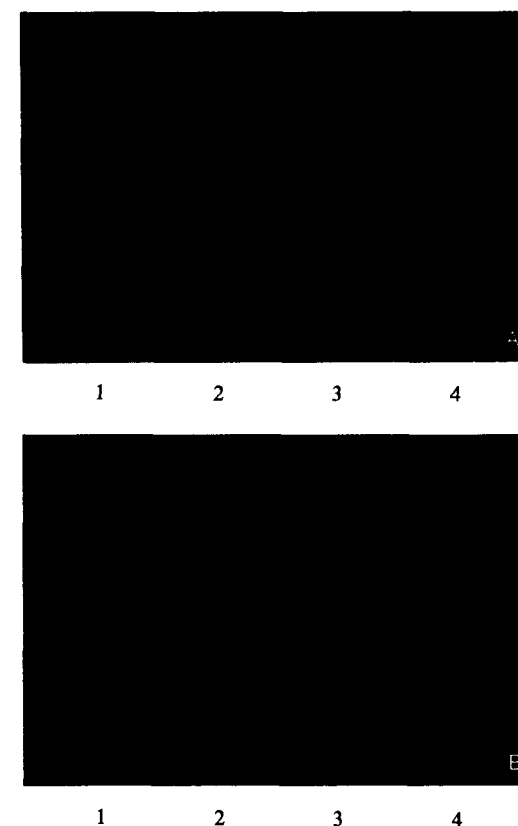


Fig. 1: Chromatograms of two natural product extracts and associated reference substances A. before and B. after application of BENEDICT's reagent.

Track 1: sinensetin (hR_f 90–95), scopoletin (hR_f 50–55); track 2: Extr. *Urticae* (extract of stinging nettle leaves); track 3: *Orthosiphon* extract; track 4: mixture of stinging nettle and *Orthosiphon* extracts.

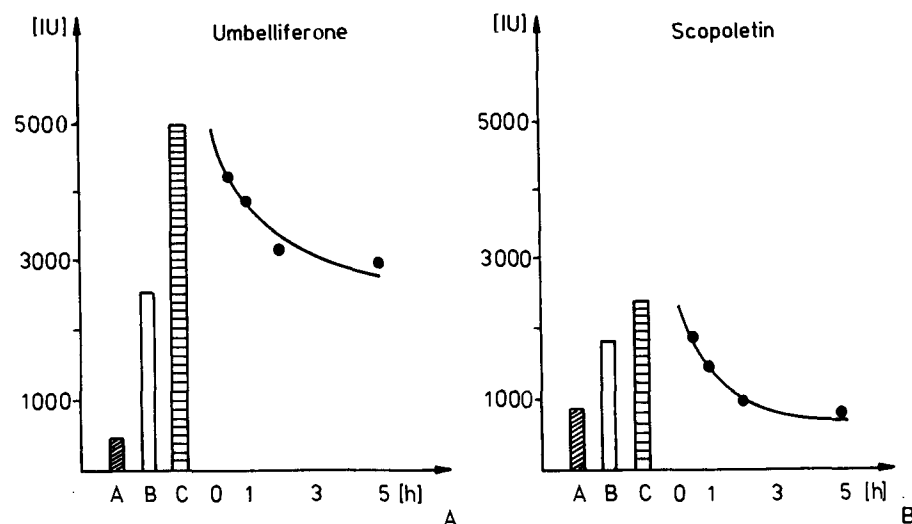


Fig. 2: Increase in fluorescence of the genuine fluorescence (A) by treatment with BENEDICT's reagent (B) and immersion in a paraffin solution (C) and reduction of emission intensities with time for the two cumarins umbelliferone and scopoletin (curves).

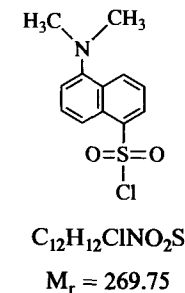
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Dansyl Chloride Reagent

Reagent for:

- Primary and secondary amines [1–4]
e.g. cactus alkaloids [1–5]
such as hordenine, tyramine, synephrine
- Amino acids
- Phenols

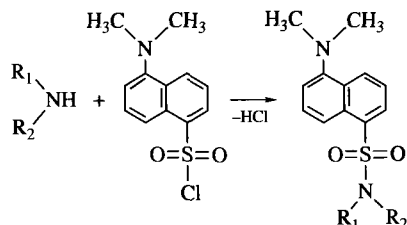


Preparation of the Reagent

- Dipping solution I** Dissolve 2 to 5 g sodium carbonate in 50 ml water and make up to 100 ml with methanol [6].
- Dipping solution II** Dissolve 100 mg dansyl chloride (5-(dimethylamino)-naphthalene-1-sulfonyl chloride) in 100 ml ethanol [6].
- Spray solution** Dissolve 50 mg dansyl chloride in 100 ml acetone [3–5].
- Storage** Dipping solution I can be stored for a longer period. Dipping solution II and the spray solution should be made up fresh daily and protected from light.
- Substances** 5-(Dimethylamino)-naphthalene-1-sulfonyl chloride
Sodium carbonate, anhydrous
Methanol
Ethanol (96%)

Reaction

Dansyl chloride that exhibits a blue intrinsic fluorescence, reacts with many amines and phenols to yield derivatives with fluorescence of another color.



Method

The chromatogram is freed from mobile phase, immersed in dipping solution I for 1 s or sprayed homogeneously with it, dried in a stream of warm air and immersed immediately after cooling for 1 s in dipping solution II or sprayed homogeneously with the spray solution and then heated to 110 °C for 2 min.

Under long-wavelength UV light ($\lambda = 365 \text{ nm}$) yellow-orange fluorescent chromatogram zones are observed on a pale light-blue fluorescent background.

Note: Tertiary amines do not react with dansyl chloride and can be detected by spraying afterwards with WAGNER's reagent [1]. The detection limits for amines are in the lower nanogram range.

The reagent can be used on silica gel, kieselguhr, Si 50000, aluminium oxide and RP layers; amino phases are unsuitable.

Procedure Tested

Biogenic Amines [6]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Ethyl acetate – 1-propanol – ammonia solution (25% (12+9+3)).
Migration distance	6 cm
Running time	20 min

Detection and result: The chromatogram was freed from mobile phase (the ammonia must be removed completely) and immersed in dipping solution I for 1 s, dried in stream of warm air for 2 min and immersed immediately after cooling in dipping solution II for 1 s and then heated to 110 °C for 2 min.

Phenylethylamine (hR_f 60–65), tyramine (hR_f 45–50), serotonin (hR_f 35–40) and histamine (hR_f 20–25) yielded yellow-orange fluorescent zones on a pale light-blue fluorescent background under long-wavelength UV light ($\lambda = 365 \text{ nm}$).

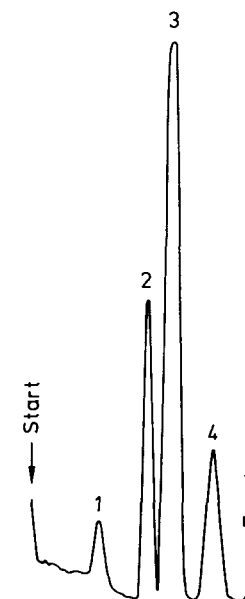


Fig. 1: Fluorescence scan of a chromatogram track with a mixture of biogenic amines with 1 µg substance per chromatogram zone: 1 = histamine, 2 = serotonin, 3 = tyramine, 4 = phenylethylamine.

In situ quantitation: The fluorimetric quantitation was carried out in long-wavelength UV light at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 560$ nm (Fig. 1).

It is not recommended that the chromatogram then be treated with liquid paraffin – n-hexane (1+4) since the intensity of the pale light blue fluorescent background is also increased, so that the difference in emission of the chromatogram zones is reduced.

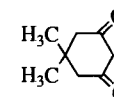
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Dimedone–Phosphoric Acid Reagent

Reagent for :

- Ketosugars [1–4]
e.g. fructose, sucrose, raffinose, lactose
- Aryl- and heteroarylpropionic acids
e.g. flurbiprofen, ketoprofen [5]



$$M_r = 140.18$$

Dimedone



$$M_r = 98.00$$

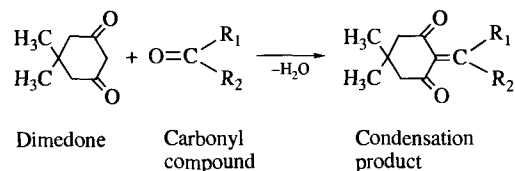
Phosphoric acid

Preparation of the Reagent

Dipping solution	Dissolve 0.3 g dimedone (5,5-dimethylcyclohexane-1,3-dione) in 90 ml ethanol and mix with 10 ml <i>ortho</i> -phosphoric acid (85% [1, 5]).
Storage	The dipping solution may be stored for an extended period.
Substances	Dimedone Ethanol <i>ortho</i> -Phosphoric acid (85%)

Reaction

Dimedone reacts with carbonyl compounds with the elimination of water yielding the condensation product [1]. The reaction is specific for ketoses; aldoses do not react or only weakly [6].



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 4 s or homogeneously sprayed with it until the layer begins to be transparent and then heated to 110°C for 15 to 20 min, after briefly drying in a stream of cold air.

Yellow chromatogram zones are formed on a colorless background; these zones mostly fluoresce blue when excited with long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: In the case of aryl- and heteroarylpropionic acids the chromatograms are irradiated with unfiltered UV light for 30 min before application of the reagent [5]. The chromatograms can then be immersed in a solution of liquid paraffin – *n*-hexane (1+2) in order to stabilize and enhance the fluorescence [5].

The detection limits for aryl- and heteroarylpropionic acids are in the lower nanogram range [5, 7]. In the case of ketosugars 10–40 ng substance can be detected per chromatogram zone [1].

The reagent can, for instance, be used on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Flurbiprofen and Ketoprofen [5]

Method	Ascending, one-dimensional development in a trough chamber without filter paper lining. The development was commenced 30 min after charging the chamber with 5 ml mobile phase.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	<i>n</i> -Hexane – diethyl ether – 1-butanol – ethyl acetate (65+15+11+9).
Migration distance	6 cm
Running time	20 min

Detection and result: The chromatogram was freed from mobile phase for 10 min in a stream of cold air, irradiated for 30 min with unfiltered UV light, then immersed in the dipping solution for 4 s and finally heated to 110°C for 15 min. The chromatogram

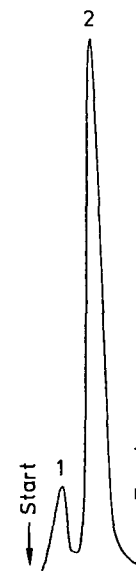


Fig. 1: Fluorescence scan of a chromatogram track with 500 ng each substance per chromatogram zone: 1 = ketoprofen, 2 = flurbiprofen.

was then immersed in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s in order to stabilize and enhance the fluorescence by a factor of about 2.

On excitation with long-wavelength UV light ($\lambda = 365$ nm) ketoprofen (hR_f 35–40) and flurbiprofen (hR_f 50–55) appeared as yellow or blue fluorescent chromatogram zones on a pale blue fluorescent background. The detection limits of, for instance, flurbiprofen were 10 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric evaluation was carried out under long-wavelength UV light at $\lambda_{exc} = 313$ nm and the fluorescence emission was measured at $\lambda_{fl} > 390$ nm (cut off filter FL 39).

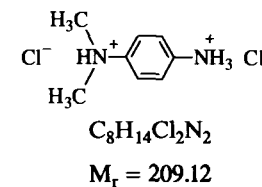
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N,N-Dimethyl-1,4-phenylenediamine Reagent (Wurster's Red Reagent)

Reagent for:

- Peroxides
 - e. g. alkyl hydroperoxides and their esters, dialkyl and diacyl peroxides, ketone peroxides [2]
 - cumol hydroperoxide [2, 3]
 - nonanoyl peroxide, *tert*-butyl perbenzoate [3]
 - sterol hydroperoxides [4]
 - linoleic acid hydroperoxides [5]
 - pregnen-17 α -hydroperoxides [6]
- Halogen-containing substances
 - e. g. chlorine-containing insecticides [1, 7]
 - such as aldrin, dieldrin, DDT, perthane, hexachlorocyclohexane, methoxychlor [7]
 - e. g. bromine-containing hypnotics [1]
 - e. g. antimicrobials
 - such as triclosan (Irgasan) [8]
- Steroids [4]
 - e. g. Δ^4 - and Δ^5 -3-ketosteroids, Δ^4 -ketosteroid- α -ketols
 - cholest-5-en-3 β ,7 α (or 7 β)-diol
- Triazines [9, 10]

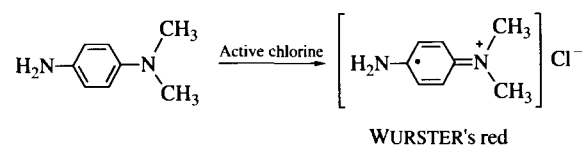


Preparation of the Reagent

Solution I	Dissolve 1 g <i>N,N</i> -dimethyl-1,4-phenylenediammonium dichloride (<i>N,N</i> -DPDD) in 100 ml ethanol at 40 °C.
Solution II	Dilute 10 ml ethanolic sodium ethylate solution (20%) to 100 ml with ethanol.
Dipping solution	Mix equal volumes of solutions I and II; filter off the precipitate that forms.
Spray solution	<p><i>For peroxides:</i> Dissolve 1 g <i>N,N</i>-DPDD in a mixture of 50 ml methanol, 50 ml water and 1 ml glacial acetic acid [4, 6].</p> <p>Reagents with other compositions are also in use, e.g. 1.5 g <i>N,N</i>-DPDD in methanol – water – glacial acetic acid (128+25+1) [1, 2] or 0.1 % <i>N,N</i>-DPDD in chloroform – glacial acetic acid – water (50+50+10) [5].</p> <p><i>For halogen-containing compounds:</i> Dissolve 0.5 g <i>N,N</i>-DPDD in a mixture of 50 ml solution II and 50 ml ethanol [1, 8].</p>
Storage	Solution I and the dipping solution should always be freshly made up. The spray solution and solution II can be stored for a longer period in the refrigerator.
Substances	<i>N,N</i> -Dimethyl-1,4-phenylenediammonium dichloride Sodium ethylate (20% in ethanol) Ethanol

Reaction

Peroxides oxidize *N,N*-DPDD to WURSTER's red, a semiquinone diimine derivative [4]. Similarly WURSTER's red is also produced from *N,N*-DPDD by reaction with halogen-containing substances in the presence of sodium ethylate and UV light and by reaction with the chlorinated triazines produced by reaction with chlorine [7].



Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and then dried in a stream of cold air. Triazines must be converted to chlorinated derivatives by exposing the chromatogram to chlorine gas (see "Procedure Tested") before application of the reagent.

In the case of halogen-containing substances (e.g. insecticides) the chromatogram is moistened by spraying with water after treatment with the reagent and then exposed to unfiltered UV light for ca. 1 min [1, 7] or to sunlight for 30 min [8].

Peroxides yield reddish-pink to purple-red chromatogram zones on a pale pink-colored background [2, 4, 5] and halogen-containing substances dirty violet, ultramarine-grey to greenish zones [7, 8]. Triazines yield intense grey to brown zones on a light brown background, observed from the back of the plate they are intense purple-red. A series of steroids also react; e.g. Δ^4 - and Δ^5 -3-ketosteroids produce a yellow to brown color and Δ^4 -3-ketosteroid- α -ketols (e.g. cortisone) orange to pink-orange zones while the two cholest-5-en-3 β ,7 α -(and 7 β)-diols only react slowly to yield a blue color [4].

Note: The contrast between the colored zones and the layer background can be improved by warming the chromatogram gently [4]. Di-*tert*-butyl peroxide does not react [3]. *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (*q.v.*) can also be used instead of *N,N*-DPDD for the detection of peroxides [3]. The spray solution for peroxides gradually turns dark red in color but it still retains its ability to react for several weeks [4].

The detection limits for peroxides are about 500 ng or with *N,N,N',N'*-tetramethyl-phenylenediamine reagent 50 ng substance per chromatogram zone [4]. The detection limits for insecticides are 5 μ g per chromatogram zone in the most unfavorable cases [7].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Triazines [10, 11]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F _{254s} (MERCK, RIEDEL-DE-HAEN); before application of the samples the layer is immersed in 2-propanol for 12 hours (preferably overnight) and then activated on a hot plate for 15 min at 110°C.
Mobile phase	1. Pentane – chloroform – acetonitrile (50+40+10) (Fig. 1). 2. Cyclohexane – dichloromethane – dioxan – tetrahydrofuran (80+10+5+5) (Fig. 2).
Migration distance	7 cm
Running time	25 min

Detection and result: The chromatogram was first dried in a stream of cold air for 5 min and then for 15 min on a hot plate at 60°C. It was then exposed for 1 min to an atmosphere of chlorine gas, that had been generated in a trough chamber by pouring 5 ml hydrochloric acid (ca. 20%) onto 0.5 g potassium permanganate placed in a small beaker (waiting time ca. 2 min before insertion of the plate). The chromatogram was then freed from excess chlorine for exactly 5 min in a stream of cold air (prolonged ventilation makes the result worse), immersed in the dipping solution for 3 s and dried for 5 min in a stream of cold air.

The substances methoprotryn (hR_f 30–35), desmetryn (hR_f 40–45), ametryn (hR_f 55–60), prometryn (hR_f 65–70) and dipropetryn (hR_f 70–75) separated using mobile phase 1 and the components cyanazine (hR_f 20–25), simazine (hR_f 30–35), atrazine (hR_f 35–40), terbutylazine (hR_f 45–50) and anilazine (hR_f 60–65) chromatographed with mobile phase 2 all yielded intense grey to brown-colored zones on a light brown background, that appear intense purple-red when viewed from the back of the plate (WURSTER's red).

In situ quantitation: The photometric evaluation was carried out in reflectance at a wavelength of $\lambda = 460$ nm (Fig. 1) and 545 nm (Fig. 2). The detection limits lay at 15 ng substance per chromatogram zone.

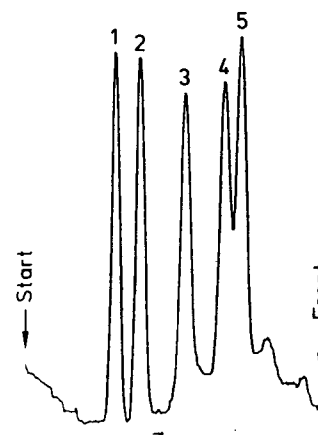


Fig. 1

Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone 1 = methoprotryn, 2 = desmetryn, 3 = ametryn, 4 = prometryn, 5 = dipropetryn.



Fig. 2

Fig. 2: Reflectance scan of a chromatogram track with 100 ng substance per chromatogram zone 1 = cyanazine, 2 = simazine, 3 = atrazine, 4 = terbutylazine, 5 = anilazine.

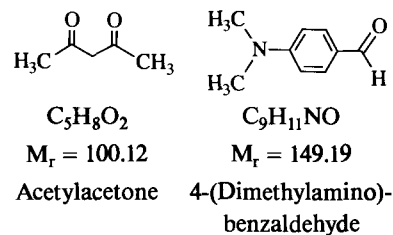
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4-(Dimethylamino)-benzaldehyde–Acetylacetone–Reagent (Morgan-Elson Reagent)

Reagent for:

- Amino sugars
e. g. glucosamine, galactosamine



Preparation of the Reagent

Solution I	Mix 5 ml potassium hydroxide solution (50%) with 20 ml ethanol [1].
Solution II	Mix 0.5 ml acetylacetone and 50 ml 1-butanol [1].
Reagent solution	Mix 0.5 ml solution I with 10 ml solution II immediately before use [1, 2].
Dipping solution	Dissolve 1 g 4-(dimethylamino)-benzaldehyde in 30 ml ethanol, add 30 ml hydrochloric acid (37%) and dilute with 180 ml 1-butanol [2].

Spray solution	Dissolve 1 g 4-(dimethylamino)-benzaldehyde in 30 ml ethanol and add 30 ml hydrochloric acid (37%) [1].
Storage	The reagent solution should always be made up fresh [1], since can be stored for only a few hours.
Substances	4-(Dimethylamino)-benzaldehyde Acetylacetone Potassium hydroxide Ethanol 1-Butanol Hydrochloric acid, fuming (37%)

Reaction

The mechanism of the reaction has not been elucidated.

Method

The dried chromatograms are dipped in the reagent solution for 3 s or spray homogeneously with it and then heated to 105 °C for 5 min. After cooling to room temperature the chromatograms are then immersed in the dipping solution homogeneously sprayed with the spray solution. They are finally dried at 90 °C for 5 min [1, 2].

Red to brown chromatogram zones are produced on a colorless to yellow background [1].

Note: The reagent is not very sensitive. Detection is also evidently affected by external influences (pH, temperature, heating time etc.), which have an effect on the detection sensitivity and on the colors of the chromatogram zones [3].

If the order of application of the reagents is reversed and all other conditions kept the same, lemon-yellow chromatogram zones are produced on a pale yellow background.

The N-acetyl derivatives of 2-amino hexoses give a reaction even in the absence of acetylacetone [4].

The detection limits for amino sugars are ca. 0.5 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr, Si 50000 and cellulose layers as well as on RP, CN, Diol and NH₂ layers.

Procedure Tested

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	2-Propanol – ethyl acetate – ammonia solution (32%) (10+10+10) [5].
Migration distance	8 cm
Running time	90 min

Detection and result: The chromatogram was dried in a stream of warm air and immersed for 3 s in the reagent solution and then heated to 105 °C for 5 min. After cooling to room temperature it was immersed in the dipping solution for 3 s and then dried at 90 °C for 5 min.

Galactosamine (*hR_f* 30–35) and glucosamine (*hR_f* 35–40) produce brownish-red chromatogram zones on a yellow background.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 400$ nm (Fig. 1).

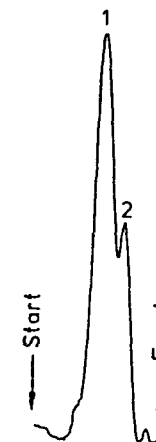


Fig 1: Reflectance scan of a chromatogram track with 5 µg galactosamine (1) and 1 µg glucosamine per chromatogram zone.

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4-(Dimethylamino)-benzaldehyde–Acid Reagents

A whole series of derivatization reagents contain 4-(dimethylamino)-benzaldehyde as a fundamental component. They differ in the type and concentration of the mineral acid components used in their preparation. Other components of the reagent generally play a minor role.

The two most commonly used dimethylaminobenzaldehyde reagents bear the names of their “inventors” who first described the acid component used. They are known as

EHRlich’s reagent or VAN URK’s reagent

depending on whether hydrochloric acid or sulfuric acid is used in the reagent. Many publications do not follow this naming system — this is particularly evident where the authors give the composition of the dimethylaminobenzaldehyde reagent employed — so that the reaction names for EHRlich’s and VAN URK’s reagent have not always been cited correctly in the past.

The fact that many publications just refer to EHRlich’s or VAN URK’s reagent without any other reference to the actual composition, necessarily means that the methods in such publications present a probable source of lack of reproducibility when an attempt is made to reproduce the results.

A publication by EHMANN reveals how confused the situation is; here the introduction correctly describes

EHRlich’s reagent as 4-(dimethylamino)-benzaldehyde — hydrochloric acid and

VAN URK’s reagent as 4-(dimethylamino)-benzaldehyde — sulfuric acid,

but the experimental section incorrectly refers to a solution of 4-(dimethylamino)-benzaldehyde in hydrochloric acid/ethanol as VAN URK’s reagent [1].

It can be concluded from the publications of ROHDE [2] and FREUND and LEBACH [3, 4] that it was EHRlich who first suggested the use of 4-(dimethylamino)-benzaldehyde in the presence of hydrochloric acid for color reactions with “methylketols” that was found also to apply to indole derivatives [5]. AUTERHOFF [6] designated a reagent for urobilinogen, consisting of a solution of 2 g 4-(dimethylamino)-benzaldehyde in 20 percent hydrochloric acid, correctly as EHRlich’s solution. Hence, it is essentially correct to refer to all reagents which contain these components as EHRlich’s reagent.

The designation VAN URK’s reagent can be traced back to a publication in 1929 [7] which describes the detection of ergot alkaloids with 4-(dimethylamino)-benzaldehyde in aqueous solution by cautiously underlayering with concentrated sulfuric acid.

AUTERHOFF described a reagent made up of 4-(dimethylamino)-benzaldehyde, sulfuric acid with additional iron(III) ions as “VAN URK’s reagent solution”. A more correct name would have been VAN URK-SALKOWSKI reagent, since the SALKOWSKI reagent (iron(III) chloride/sulfuric acid) has been used alongside the VAN URK’s reagent [8].

Similar errors have been perpetuated in the literature until the present day (cf. [9–11]).

Although more than 150 publications have been reviewed the “dimethylaminobenzaldehyde monographs” that follow only take account of and cite those where there is an unequivocal citation of precise formulations — with respect to the acid used in the reagent — so that they can be assigned to the appropriate named reaction. This procedure was justified in view of the results obtained for the examples tested for the reagent monographs, for there are differences between the two reagents. EHRlich reagent is usually more sensitive and yields better method standard deviations on in situ evaluation [12]. However, the plate background is yellow in color while it remains white after the use of VAN URK’s reagent [13], or acquires a slight gray color [12]. Furthermore, the layer background does not discolor with standing after the use of the VAN URK reagent as is the case with EHRlich’s reagent [14]. Nevertheless the VAN URK reagent has been used less frequently for TLC in the past but was primarily employed in solution photometry [13], e.g. for the characterization of substances on the basis of aldol condensations. This also applies to determinations made after elution of scraped off chromatogram zones [11, 15–18].

In rare cases — such as the detection of nafazatrom [19] — 4-(dimethylamino)-benzaldehyde is used without the addition of any acid.

References

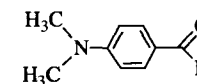
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4-(Dimethylamino)-benzaldehyde– Acetic Acid–Phosphoric Acid Reagent (EP Reagent)

Reagent for:

- Terpene and sesquiterpene derivatives
e.g. azulenes, proazulenes [1–9]
such as chamazulene, or matricin
e.g. bisabolol [2, 3]
- Sesquiterpene esters
e.g. cinnamoylchinaldiol, -echinaxanthol, dihydroxynardol



$C_9H_{11}NO$	CH_3COOH	H_3PO_4
$M_r = 149.19$	$M_r = 60.05$	$M_r = 98.00$
4-(Dimethylamino)- benzaldehyde	Acetic acid	Phosphoric acid

Preparation of the Reagents

- Dipping solution** Dissolve 0.25 g 4-(dimethylamino)-benzaldehyde in 50 ml glacial acetic acid and add 3 ml *ortho*-phosphoric acid (85%) [10].
- Spray solution** Dissolve 0.25 g 4-(dimethylamino)-benzaldehyde in a mixture of 50 g glacial acetic acid, 5 g *ortho*-phosphoric acid (85%) and 20–45 ml water [1–3, 5, 8].

Storage	The reagent solutions can be stored for months in well-sealed, brown glass bottles [1, 2].
Substances	4-(Dimethylamino)-benzaldehyde Acetic acid <i>ortho</i> -Phosphoric acid

Reaction

The mechanism of the reaction has not yet been elucidated.

Method

The chromatograms are dried in a stream of warm air and then immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution and then heated to 80–110°C for 10–20 min [1, 2, 10].

Generally blue to violet chromatogram zones are formed on a colorless background. Azulene appears pale green and proazulenes produce gray, violet, brown, orange or green chromatogram zones [2].

Note: The EP reagent can be used for the specific detection of matricin in chamomile extract [1].

The detection limits per chromatogram zone are 250 ng for bisabolol and 40 ng for bisabolol dioxide [10].

The reagent can be employed, for example, on silica gel, kieselguhr, Si 50000, CN, diol, RP and cellulose layers.; NH₂ and polyamide phases are not suitable since the whole background acquires a yellow color, and the substances do not react [10].

Procedure Tested

Chamomile oil [10]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 (MERCK).
Mobile phase	Toluene – ethyl acetate (7+3).
Migration distance	6 cm
Running time	7 min

Detection and result: The chromatogram was dried in a stream of warm air, immersed in the dipping solution for 2 s and then heated to 110°C for 20 min.

Bisabolol oxide (*hR_f* 40–45) appeared as pink and bisabolol (*hR_f* 65–70) as mauve-colored chromatogram zones on a pale yellow background. The detection limits per chromatogram zone were 40 ng for bisabolol oxide and 250 ng for bisabolol.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 515$ nm (Fig. 1).

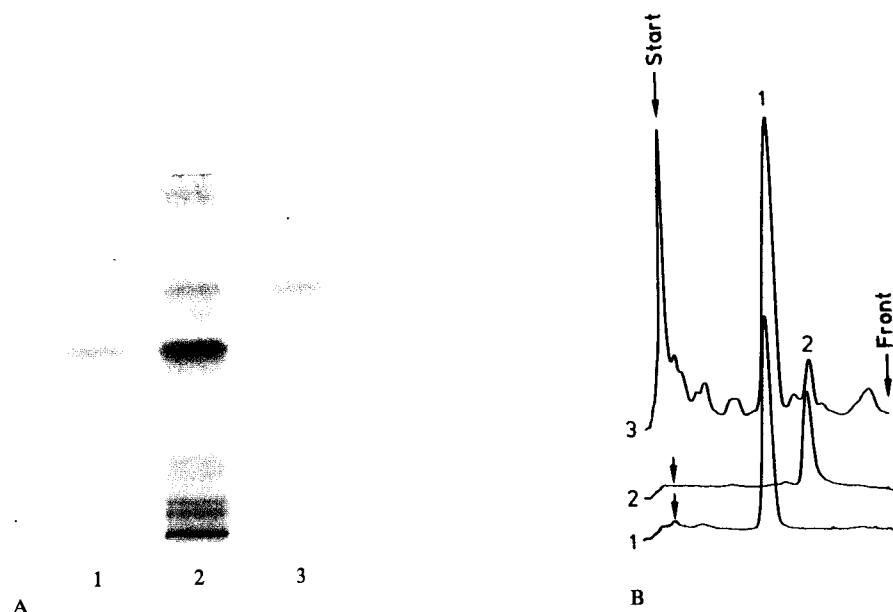


Fig 1: Chromatogram of a chamomile flower extract and of chamomile oil components (A) and reflectance scans (B) of reference tracks with 3.75 µg bisabolol oxide (1) and 9.5 µg bisabolol (2) and a chromatogram track with chamomile flower extract (3).

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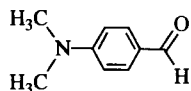
4-(Dimethylamino)-benzaldehyde-Hydrochloric Acid Reagent (Ehrlich's Reagent)

Reagent for:

- Indole derivatives [1, 3]
 - e.g. ergot alkaloids [2, 4–12]
 - such as ergotamine, ergocristine, ergometrine, ergocornine dihydroergosine,, lysergamide, isolysergamide
 - e.g. ergolinecarboxylic acids [13–16]
 - e.g. *clavine* alkaloids [4, 6, 17, 18]
 - such as agroclavine, chanoclavine, penniclavine
 - e.g. harpagophytum alkaloids [19]
 - such as harpagoside
 - e.g. hallucinatory drugs
 - such as LSD [7, 9, 12], psilocybin, psilocin [12, 20]
 - e.g. auxins [21–23]
 - such as 5-hydroxyindole-3-acetic acid, indole-3-acetic acid and their esters
 - e.g. tryptophan derivatives [2, 22–29]
 - such as tryptophan, tryptamine, serotonin, tryptophol, N-carbamyltryptophan
 - e.g. yohimbine alkaloids [30]
 - such as ajmalicine, rauniticine
 - e.g. pyrrolizidine alkaloids [31]
 - such as symphytine N-oxide and echimidine N-oxide
 - e.g. peramine [32]
- Amines [1]
 - e.g. primary aromatic amines [33–36]
- Urea and thiourea derivatives [28, 29, 37]
 - e.g. urea, thiourea
- Drug substances [38, 39]
 - e.g. sulfonamides [40–42]
 - such as sulfanilamide, sulfanilthiocarbamide, sulfathiazole

Reagent for:

- Mycotoxins
e.g. cyclopiazonic acid [43], PR toxin or PR imine [44]
diacetoxyscirpenol [45]
- Pesticides
e.g. phenylcarbamate and phenylurea herbicides [34, 36]
such as asulam, chlorbromuron, chlortoluron, diuron, linuron
- Explosives [35]
e.g. tetryl, TATB(1,3,5-triamino-2,4,6-tetranitrobenzene)
- Carbapenem antibiotics [46]
- Gangliosides [47, 48]
e.g. GM1, GM2, Fuc-GM1, GT1 b, GD1 a, GD1 b
- Bitter substances
e.g. limonin [49], nomilin [50]
- Pyridine derivatives
e.g. citrazinic acid, citrazinamide [51]



HCl
M_r = 36.46
Hydrochloric
acid

C₉H₁₁NO
M_r = 149.19
4-(Dimethylamino)-
benzaldehyde

Preparation of the Reagent

Dipping solution Dissolve 300–500 mg 4-(dimethylamino)-benzaldehyde in 25–40 ml methanol and treat with cooling with 10 ml hydrochloric acid (32%); the temperature should not fall below 20 °C or rise above 40 °C [52, 53].

Alternatively combine a solution of 300 mg 4-(dimethylamino)-benzaldehyde with a mixture of 54 ml 1-butanol, 9 ml ethanol and 9 ml conc. hydrochloric acid [46].

Spray solution Dissolve 1–5 g 4-(dimethylamino)-benzaldehyde in a mixture of 50 ml hydrochloric acid (25%) and 50 ml ethanol (96% or 100%) [2, 9, 11, 12, 21, 22,, 27, 32, 42], methanol [3, 10, 54] or 2-propanol [24].

Alternatively make up a stock solution of 10 g 4-(dimethylamino)benzaldehyde in concentrated hydrochloric acid and dilute one part by volume with 4 to 10 parts by volume acetone immediately before spraying [20, 25, 26, 28, 29, 44].

Storage

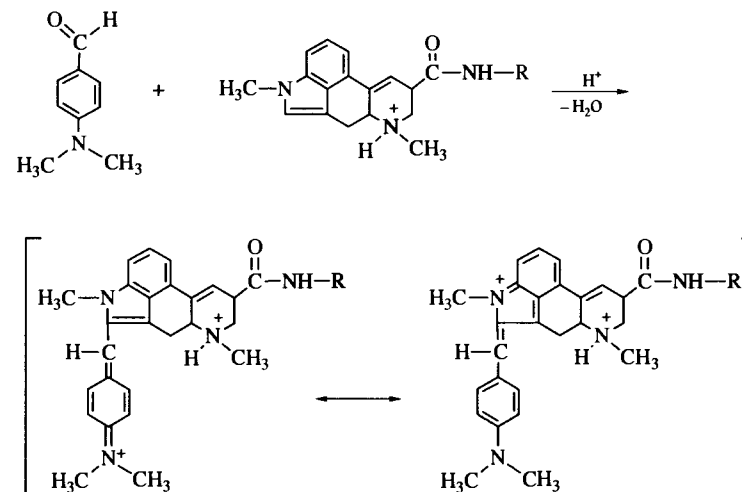
The reagent solutions may be stored for several weeks [4, 53]. The spray solution that has been diluted with acetone is not stable and should therefore always be made up fresh [28].

Substances

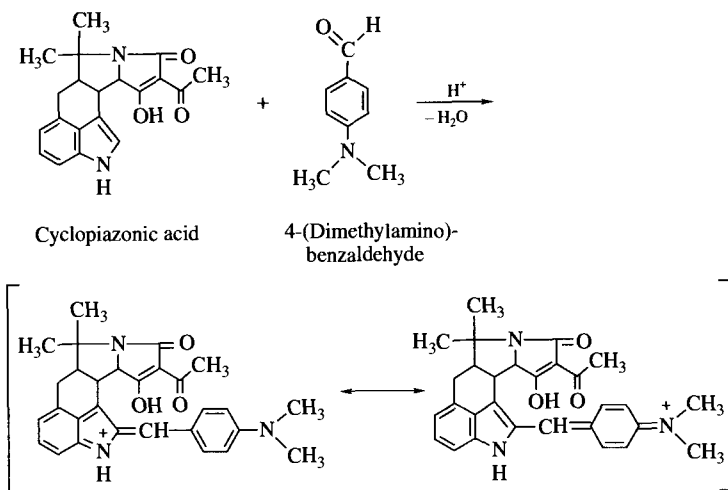
4-(Dimethylamino)-benzaldehyde
Hydrochloric acid (32%)
Hydrochloric acid (25%)
Methanol
Ethanol
Ethanol (96%)
1-Butanol

Reaction

Electrophilic substitution, e.g. of the 2-position of the indole ring, followed by the elimination of water leads to the formation of cyanin dyes from ergot alkaloids [53]



The same applies to cyclopiazonic acid [43].



Method

The chromatograms are dried in a stream of warm air, then immersed in the dipping solution for 2 s to 20 s or homogeneously sprayed with the spray solution until the layer begins to appear transparent [21, 52, 53]. After allowing the chromatogram to stand for a few minutes it is then heated to 50–120 °C for 2–20 min [12, 24, 47, 52–54]. In the case of gangliosides the chromatograms are covered with a glass plate during heating [47, 48].

Chromatogram zones of different colors (yellow, orange, red, brown, green, blue) are formed – mainly within a few minutes even before heating – on an almost colorless to slightly yellow background [2, 4, 21, 22, 52–54].

For example, ergot alkaloids produce without exception blue chromatogram zones, while clavine alkaloids primarily produce green colors [4]. Urea derivatives and primary aromatic amines yield yellow chromatogram zones [28, 33, 34, 36, 37] and PR toxin and PR imine emit intense blue fluorescence on excitation with long-wavelength UV light ($\lambda = 365 \text{ nm}$) [44].

Note: Several variants of the reagent have been described in the literature. Thus chromatograms can be sprayed with a solution of 4-(dimethylamino)-benzaldehyde in

cyclohexane, ethanol or 1-butanol and then exposed to hydrochloric acid vapor [5, 8, 13–16, 43, 44, 49, 50]. Other variants of the reagent involve the addition of a drop of iron(III) chloride solution (10%) [4] or recommend treatment of the chromatogram afterwards with sodium nitrite (1% aqueous) to stabilize the colors [6]. In exceptional cases 4-(dimethylamino)-benzaldehyde reacts alone without the addition of other components to the reagent [55]. The 4-(dimethylamino)-benzaldehyde in the reagent can be replaced by 4-(diethylamino)-benzaldehyde [35]. In the case of pyrrolizidine alkaloid the chromatogram is sprayed with acetic anhydride – petroleum ether – benzene (1+4+5) and heated to 95 °C for 10 min before being treated with EHRICH's reagent [31]. If zinc powder is incorporated into the layers it is also possible to detect nitro aromatics [35].

Some substances only react slowly at room temperature [20, 22]. The colors that appear initially generally alter over a period of a few hours and then remain stable for a virtually unlimited period [21]. The addition to the reagent of small quantities of oxidizing agents (iron(III) salts, hydrogen peroxide) has been reported to intensify the color tone [2, 4]; the same is also reported to occur if the treated chromatograms are afterwards exposed to UV light or to the vapors of aqua regia or nitric acid [2, 12].

4-(Dimethylamino)-benzaldehyde – hydrochloric acid reacts less sensitively than 4-(dimethylamino)-cinnamaldehyde – hydrochloric acid in the detection of indole derivatives, but the former is better for differentiation of substances on account of the multiplicity of different color shades produced.

The detection limits per chromatogram zone are 4–20 ng substance for aniline derivatives [52] and 3–100 ng substance for indole derivatives [2, 4, 5, 32, 53]. But some substances, e.g. dihydroergosine [11] and PR toxin and PR imine [44] can be detected in quantities smaller than 1 ng.

The reagent can be employed, for example, on aluminium oxide, silica gel, silver nitrate impregnated silica gel, kieselguhr, Si 50000 and cellulose layers; RP and CHIF phases are also suitable.

Procedure Tested 1

Aniline Derivatives [52]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).

Mobile phase Toluene
Migration distance 8 cm
Running time 25 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air, immersed in the dipping solution for 2 s, dried briefly in a stream of warm air and then heated on a hot plate to 110 °C for 2 min.

2,4-Dimethylaniline (hR_f 5–10), 4-chloroaniline (hR_f 10–15), 3-chloroaniline (hR_f 20–25), 4-chloro-2-nitroaniline (hR_f 30–35), 2-chloroaniline (hR_f 35–40) and diphenylamine (hR_f 70–75) appeared as yellow chromatogram zones on a pale yellow background. The detection limits were between 4 ng (4-chloroaniline) and 20 ng (diphenylamine) substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 435$ nm (Fig. 1).

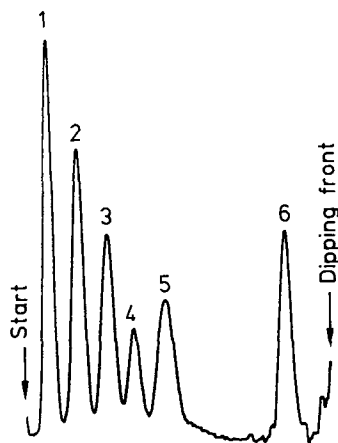


Fig 1: Reflectance scan of a chromatogram track with 100 ng each of 4-chloroaniline (2) and 3-chloroaniline (3) and 200 ng 2,4-dimethylaniline (1), 4-chloro-2-nitroaniline (4), 2-chloroaniline (5) and diphenylamine (6) per chromatogram zone.

Procedure Tested 2

Ergot Alkaloids [53]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer HPTLC plates Silica gel 60 F₂₅₄ (MERCK).
Mobile phase Acetonitrile – 1-propanol – water (125 + 28 + 15).
Migration distance 6 cm
Running time 17 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air and immersed twice for 10 s – with brief intermediate drying in a stream of cold air – in the dipping solution and then immediately heated to 115 °C for 15 min in the drying cupboard; the TLC plate was only to be supported on its side on two metal tracks. After cooling to room temperature the chromatogram was immersed for 1 s in a solution

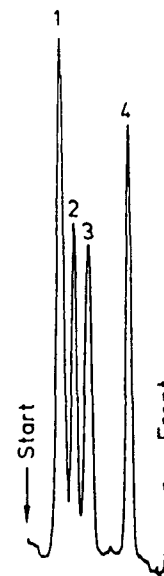


Fig 2: Reflectance scan of a chromatogram track with 90 ng of each substance per chromatogram zone; 1 = lisuride dihydrogen maleate, 2 = methysergide maleate, 3 = dihydroergotamine mesylate, 4 = ergotamine tartrate.

tion of liquid paraffin — *n*-hexane (1+2) and dried for 5 min in a stream of cold air; the purpose of this last immersion was to stabilize the reflectance signal of methysergide in particular.

Lisuride dihydrogen maleate (hR_f 30–35), methysergide maleate (hR_f 40–45), dihydroergotamine mesylate (hR_f 45–50) and ergotamine tartrate (hR_f 70–75) appeared as blue violet chromatogram zones on a yellow background. The detection limits — calculated for free base — were 3–4 ng substance per chromatogram zone.

In situ quantitation: After 1 h the absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 590$ nm (Fig. 2).

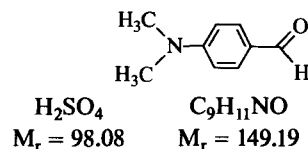
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4-(Dimethylamino)-benzaldehyde– Sulfuric Acid Reagent (Van Urk's Reagent)

Reagent for:

- Primary aromatic amines [1]
- Alkaloids
 - e.g. tropane alkaloids
such as atropine, scopolamine [2]
 - e.g. ergot alkaloids
such as ergotamine, ergocryptine, ergometrine [3, 4, 17]
 - e.g. clavine alkaloids
such as chanoclavine, agroclavine [3, 5, 6]
- Pharmaceutical active ingredients
 - e.g. sulfonamides
such as sulfasomidine, sulfadiazine, sulfamerazine [1, 7]
- Addictive drugs
 - e.g. lysergic acid diethylamide (LSD) [4, 8–10]
psilocybin, monomethyltryptamine [9]
- Indole and hydroxyindole derivatives
 - e.g. tryptophan metabolites [11]
cyclopiazonic acid (mycotoxin) [12]
4-, 5-, 6- and 7-hydroxyskatoles [13]
- Monoterpene ketones
 - e.g. menthone, pulegone, carvone, piperitenone [14]
- Carbamate pesticides
 - e.g. fenuron, monuron, linuron, carbaryl [15]



Preparation of the Reagent

Dipping solution Dissolve 1 g 4-(dimethylamino)-benzaldehyde in a mixture of 45 ml water and 5 ml sulfuric acid (95–97%) and make up to 100 ml with water [2].

Variant 1: Dissolve 2 g 4-(dimethylamino)-benzaldehyde in 45 ml methanol, add 5 ml sulfuric acid (95–97%) cautiously with cooling and make up to 100 ml with methanol [16].

Variant 2: Dissolve 0.3 g 4-(dimethylamino)-benzaldehyde in 90 ml methanol and add 10 ml sulfuric acid (95–97%) cautiously with cooling (ice water). When preparing this dipping solution the temperature should be kept within the 20–40 °C range [17].

Spray solution *For indole derivatives:* Dissolve 50 mg 4-(dimethylamino)-benzaldehyde in 1 ml conc. sulfuric acid and make up to 100 ml with 95% ethanol [7].

Variant 1: Dissolve 0.8 g 4-(dimethylamino)-benzaldehyde in a mixture of 90 ml 95% ethanol and 10 ml 98% sulfuric acid [9].

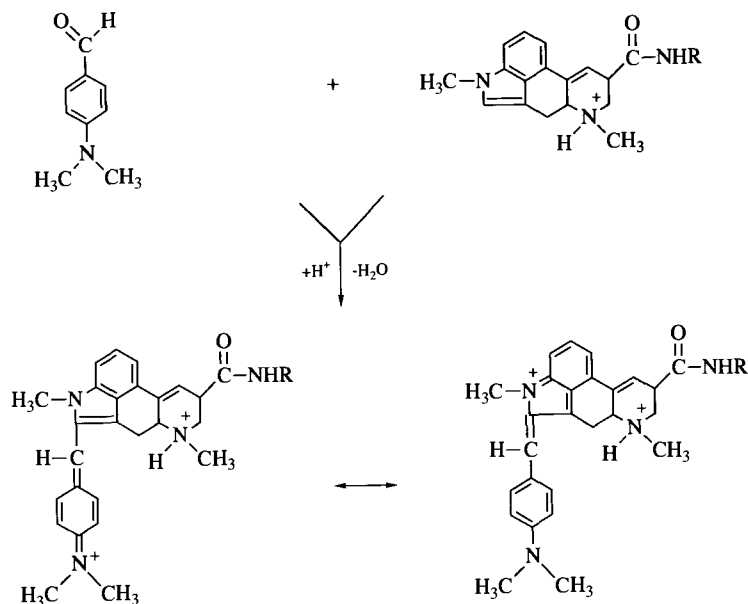
For monoterpene ketones: Dissolve 200 mg 4-(dimethylamino)-benzaldehyde in 20 ml conc. sulfuric acid [14].

Storage The reagent solutions may be stored over a prolonged period [17].

Substances 4-(Dimethylamino)-benzaldehyde
Sulfuric acid (95–97%)
Methanol
Ethanol

Reaction

4-(Dimethylamino)-benzaldehyde reacts in acidic medium, e.g. with the indole ring of cyclopiazone or ergot alkaloids and forms a cyanin dyestuff by electrophilic substitution in the 2-position followed by the elimination of water [12, 17].



Method

The chromatograms are freed from mobile phase in a stream of warm air, then either immersed briefly in one of the dipping solutions or homogeneously sprayed with one of the spray solutions, until the layer begins to be transparent. Then they are heated to 105–120°C for 10–30 min [2, 9, 14, 16, 17].

Indole derivatives yield red to blue-violet chromatogram zones on an almost colorless background [2, 3, 12]; these zones gradually fade [12]. Monoterpene ketones yield yellow-gray to red-brown chromatogram zones [14]. Hydroxyskatoles initially yield yellow chromatogram zones but these change color to gray-brown or green-brown on heating or if allowed to lie [13].

Note: The individual components of the reagent can also be applied separately one after the other [12, 15], e.g. the chromatogram is first immersed in an 8% methanolic 4-(dimethylamino)-benzaldehyde solution and then, after intermediate drying, sprayed with 25% sulfuric acid [12]. 4-(Dimethylamino)-benzaldehyde can be replaced in the reagent with 4-(dimethylamino)-cinnamaldehyde [1].

In the case of carbamate pesticides the chromatogram is heated to 150°C for 20 min after the application of the reagent. Spraying later with a solution of 2 N sodium hydroxide solution to improve the color contrast is recommended [15]. Occasionally a small amount of iron(III) chloride is added to the reagent [13].

Quantitative evaluations are best carried out ca. 15–20 min after heating the chromatogram [2].

The detection limits for sulfonamides lie at 10–50 ng [7], for lysergic acid diethylamide at 50 ng [4] and for tropane alkaloids at 50–500 ng substance per chromatogram zone [2, 16].

In the case of lysergic acid derivatives spraying with sodium nitrite solution afterwards stabilizes the colored chromatogram zones [18].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr and Si 50000 layers; cellulose layers are not suitable.

Procedure Tested 1

Tropane Alkaloids [16]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that are prewashed, before application of the samples, by developing in chloroform – methanol (50+50) to the upper edge of the plates and then dried for 30 min at 110°C.
Mobile phase	Acetone – toluene – ammonia solution (25%) (40+15+5).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air until the ammonia was completely removed (ca. 45 min), cooled in a stream of cold air for 5 min, immersed twice in the dipping solution (variant 1) for 10 s, with brief intermediate drying in a stream of cold air, and then heated to 120°C for 30 min.

Atropine (hR_f 30–35) and scopolamine (hR_f 60–65) appeared as red chromatogram zones on a reddish-gray background. The detection limits lay at 50 ng substance per spot.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at wavelength $\lambda = 500$ nm (Fig. 1).

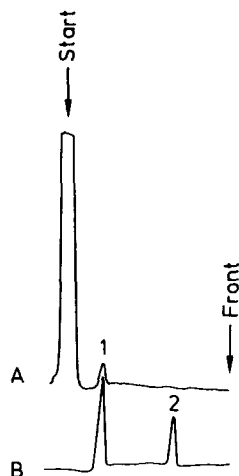


Fig. 1: Reflectance scan of a chromatogram track of an *Atropa belladonna* extract (A) and of a reference track (B) with 200 ng of both atropine (1) and scopolamine (2).

Procedure Tested 2

Ergot Alkaloids [17]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Acetonitrile – 1-propanol – water (125+28+15).
Migration distance	6 cm
Running time	17 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed twice in the dipping solution (variant 2) for 10 s with brief

intermediate drying in a stream of cold air and then heated, while still damp, to 95 °C for 1 min on a hot plate. Then, after cooling to room temperature, it was immersed for 1 s in a solution of liquid paraffin – *n*-hexane (1+2) to stabilize the reflectance signal of dihydroergotamine and dried for 5 min in a stream of cold air.

Lysuride hydrogen maleate (hR_f 30–35), methysergide maleate (hR_f 40–45), dihydroergotamine mesylate (hR_f 45–50) and ergotamine tartrate (hR_f 70–75) appeared as gray-violet chromatogram zones on a colorless background. The detection limits – calculated for the free base – were 15–30 ng substance per chromatogram zone.

In situ quantitation: After waiting for 50 min the absorption photometric evaluation in reflectance was carried out at wavelength $\lambda = 590$ nm (Fig. 2).

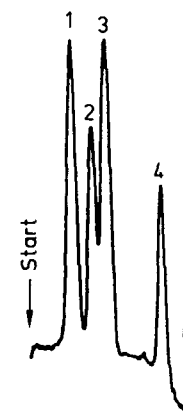


Fig. 2: Reflectance scan of a chromatogram track of 90 ng each lysuride hydrogen maleate (1), methysergide maleate (2), dihydroergotamine mesylate (3) and ergotamine tartrate (4) per chromatogram zone.

References

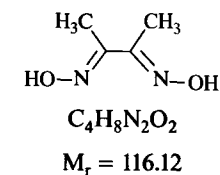
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Dimethylglyoxime Reagent (Diacetyldioxime Reagent)

Reagent for:

- Cations
 - e. g. nickel [1–7]
 - cobalt [4–7]
 - copper, iron, manganese, silver [6]
 - DMSO complexes of cobalt, nickel [8]

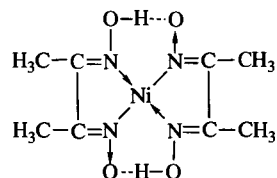


Preparation of the Reagent

Dipping solution	Dissolve 1 g dimethylglyoxime (diacetyldioxime) in 100 ml ethanol.
Spray solution	Dissolve 0.1 to 1 g dimethylglyoxime in 100 ml 96% ethanol [1, 5] or in ammonia solution [4, 8].
Storage	The reagent solutions may be stored for longer periods.
Substances	Dimethylglyoxime Ethanol Ammonia solution (25%)

Reaction

A series of metal cations (Ni, Fe, Co, Cu, Pt) form colored complexes with dimethylglyoxime in ammonia solution or weakly acidic medium.



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s or sprayed homogeneously with the spray solution, then dried in a stream of cold air and exposed to ammonia vapor in a twin-trough chamber.

Red-violet (Ni), red-brown (Co, Fe, Cu), flesh-colored (Mn) or pale gray-violet (Mn) chromatogram zones are produced on a colorless background [6, 7].

Note: The detection limits for nickel and cobalt cations are 20 ng substance per chromatogram zone [9].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr, Si 50000, RP and cellulose layers. Sodium molybdate-impregnated phases and zirconium oxide layers are also suitable [1].

Procedure Tested

Nickel and Cobalt Cations [9]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer	HPTLC plates Cellulose F _{254s} (MERCK) that have been pre cleaned by developing once to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110°C for 30 min.
Mobile phase	Ethanol – water – nitric acid (65%) (70+18+12).
Migration distance	6 cm
Running time	45 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air immersed in the dipping solution for 2 s, dried for 2 min in a stream of cold air and exposed to ammonia vapor (25% ammonia solution in the vacant trough of a twin trough chamber) for 3 min.

Nickel cations (hR_f 35–40) appeared as red and cobalt cations (hR_f 40–45) as yellow chromatogram zones on a colorless background.

The detection limits lay at 20 ng substance per chromatogram zone.

In situ quantitation: The absorption spectrophotometric measurements in reflectance were made at a mean wavelength $\lambda = 480$ nm (Fig. 1A) or at wavelength $\lambda = 450$ nm for cobalt (Fig. 1B) and $\lambda = 510$ nm for nickel (Fig. 1C).

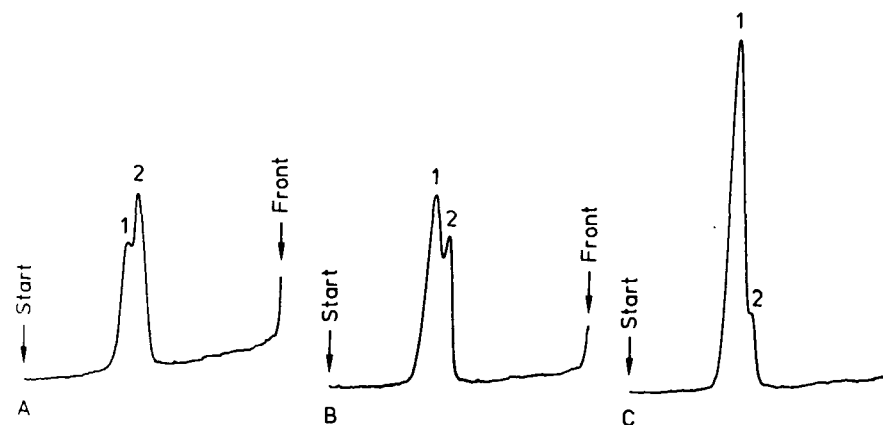


Fig. 1: Reflectance scans of a chromatogram track with 200 ng each of nickel (1) and cobalt (2) cations per chromatogram zone: Scans at $\lambda = 480$ nm (A), 450 nm (B) and 510 nm (C).

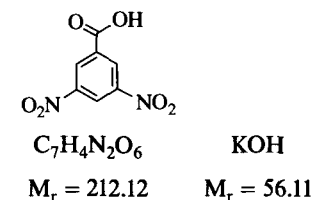
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3,5-Dinitrobenzoic Acid – Potassium Hydroxide Reagent (Kedde's Reagent)

Reagent for:

- Steroid glycosides and aglycones
e.g. cardenolide glycosides [1–7]
from *Anodendron* [8], *Digitalis* [9–12],
Strophantus [13, 14] and
Convallaria species



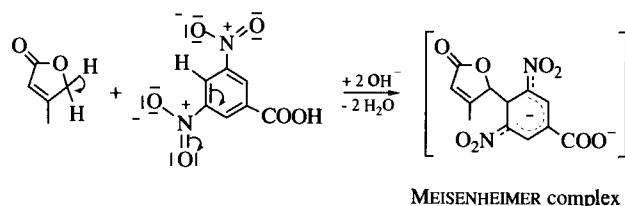
Preparation of the Reagent

Solution I	Dissolve 2 g 3,5-dinitrobenzoic acid in 100 ml methanol.
Solution II	Dissolve 5.7 g potassium hydroxide in 100 ml methanol.
Dipping solution	Dissolve 0.5 g 3,5-dinitrobenzoic acid in 50 ml ethanol (96% with gentle warming and mix with 50 ml sodium hydroxide solution ($c = 2$ mol/L) before use [17].
Spray solution	Mix equal volumes of solution I and solution II before use [1, 8, 10, 14].

Storage	Solutions I and II may be stored for extended periods.
Substances	3,5-Dinitrobenzoic acid Potassium hydroxide pellets Methanol Ethanol Sodium hydroxide solution

Reaction

The γ -lactone ring of the steroid skeleton forms an intermediate cardenolide anion in alkaline medium that nucleophilically adds to the 3,5-dinitrobenzoic acid in the position *ortho* to the two nitro groups. A mesomerically stabilized red-violet anion is produced (MEISENHEIMER complex).



Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution. Alternatively the chromatogram can first be sprayed lightly with solution I and then with an excess of solution II.

Blue to blue-violet chromatogram zones are formed on a colorless background; these gradually fade [11, 16].

Note: In the second spray potassium hydroxide solution can be replaced by sodium hydroxide solution or by a solution of 17 g benzyltrimethylammonium hydroxide in 100 ml 33 percent methanol [4]. The KEDDE reagent [15] can also be applied very suc-

cessfully to layers that have previously been treated with *p*-toluenesulfonic acid or vanillin – perchloric acid reagent [1]. The instability of the colored derivatives produced makes the reagent unsuitable for quantitative analysis [16].

The detection limits for *Convallaria* glycosides are 20 ng substance per chromatogram zone [16].

The reagent can, for instance, be used on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested I

Cardenolides [16]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK) that had been precleaned before applying the samples by developing once with methanol – chloroform (50+50) and then dried at 110°C for 30 min.
Mobile phase	Ethyl acetate – methanol – water (81+11+8).
Migration distance	10 cm
Running time	25 min

Detection and result: The chromatogram was freed from mobile phase and homogeneously sprayed with the spray solution.

The cardenolides g-strophanthin (hR_f 5–10), convallatoxin (hR_f 30–35) and k-strophanthin (hR_f 50–55) immediately formed red-violet chromatogram zones that gradually faded. Hence, the reagent was not always suitable for quantitative work.

The visual detection limits were 20 ng substance per chromatogram zone.

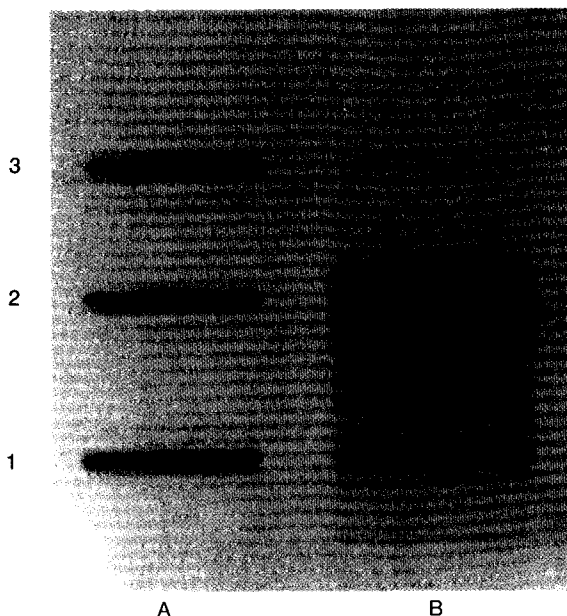


Fig. 1: Chromatograms of reference substances (A) and of a lily of the valley extract (B): 1 = g-strophanthin, 2 = convallatoxin, 3 = k-strophanthin

Procedure Tested II

Digitalis Glycosides [17]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ with concentrating zone (RIEDEL DE HAEN, MERCK).
Mobile phase	Actone – dichloromethane (60+40).
Migration distance	5 cm
Running time	8 min

Detection and result: The chromatogram was freed from mobile phase, immersed in the dipping solution for 2 s and then examined immediately after brief drying in a stream

of warm air. Digoxin (hR_f 30–35) and digitoxin (hR_f 40–45) yielded violet chromatogram zones on a colorless background. The detection limits were 50 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric scan at $\lambda_{\max} = 550$ nm had to be carried out immediately, since the colors of the derivatives only remained stable for ca. 10–15 min; exact quantitative analysis was not always possible.

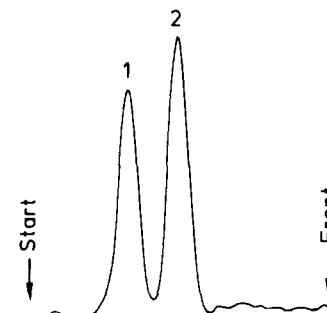


Fig. 2: Absorbance scan of a chromatogram track with 500 ng each of digoxin (1) and digitoxin (2) per chromatogram zone.

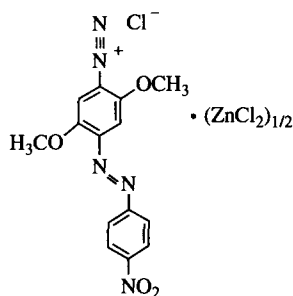
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Fast Black Salt K– Sodium Hydroxide Reagent

Reagent for:

- Amines
 - e.g. aliphatic *prim.* and *sec.* amines [1, 2]
such as α -/ β -alanine, ethylamine, diethylamine, piperidine, mescaline
desipramine, ephedrine, maprotiline, nortriptyline
 - e.g. aromatic *prim.* and *sec.* amines [1]
such as aniline, 2,4,6-trimethylaniline, bromhexine, diphenylamine,
amethocaine, procaine
 - e.g. aromatic *tert.* amines [1]
such as N,N-dimethylaniline, N,N-dimethyl-*p*-toluidine
- Phenols
 - e.g. catechin, resorcinol, hydroquinone, morphine [1]
- Salbutamol, isoprenaline, terbutaline [1]
- Tetracyclines [1]
- Aromatic N-heterocyclics
 - e.g. pyrrole, imidazole, indole [1]
- Quaternary ammonium compounds [2]
- Psychopharmaceuticals
 - e.g. chlorprothixene, haloperidol, sulpyride,
perphenazine [2]
oxaflozane and its metabolites [4]
- Analeptics, stimulants [3]
 - e.g. amphetamine, preludein, captagon [6]
methamphetamine [1, 6]
- Diethyl malonate [1]
- β -Blockers [2, 3, 5]
 - e.g. alprenolol, atenolol, oxprenolol,
propranolol, timolol, acebutolol
metoprolol, sotalol



NaOH $M_r = 40.00$
Sodium hydroxide

$(C_{14}H_{12}ClN_5O_4)_2 \cdot ZnCl_2$
 $M_r = 546.36$
Fast Black Salt K

Preparation of the Reagent

Dipping solution I Dissolve 500 mg fast black salt K (Echtschwarzsatz K, diazotized 4-amino-2,5-dimethoxy-4'-nitroazobenzene zinc double salt) in 100 ml water with heating. Filter off any undissolved components. Dilute the filtrate with methanol (1+1).

Dipping solution II Methanolic sodium hydroxide solution ($c = 0.5$ mol/L).

Spray solution I Dissolve 500 mg fast black salt K in 100 ml water; filter off any insoluble components [1–3, 5].

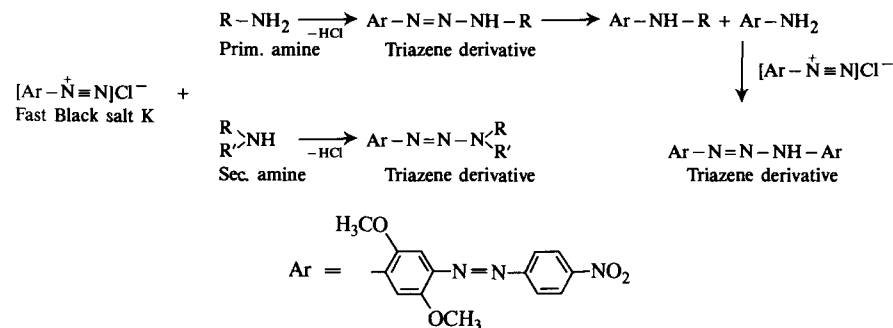
Spray solution II Sodium hydroxide solution ($c = 0.05$ mol/L) [1–3, 5].

Storage The dipping and spray solutions are stable for up to one day.

Substances
Fast black salt K
Sodium hydroxide 0.5 mol/L
Methanol

Reaction

Aliphatic primary and secondary amines primarily react with the diazonium compound fast black salt K to yield colored triazene derivatives [1] according to the following scheme:



It is also probable that there is coupling to colored derivatives in the case of aromatic amines and phenols (cf. Fast blue salt B reagent).

Method

The chromatograms are dried in a stream of warm air, sprayed homogeneously with spray solution 1, dried briefly in a stream of hot air, then sprayed lightly with spray solution 2 and finally dried in a stream of warm air. In the case of β -blockers this is followed with a further light spray with spray solution 1 [5].

Aliphatic and aromatic primary amines yield violet to violet-red chromatogram zones and aliphatic and aromatic secondary amines orange-red to brownish-red chromatogram zones on a colorless background; phenols are colored red-violet, light brown or green; pyrrole, imidazole, indole yield violet and diethyl malonate yields orange zones [1]. β -Blockers are colored orange to reddish-violet [5].

Note: Color reactions occur even before application of spray solution II [6]. Tertiary aliphatic amines and phenols with blocked *ortho* and *para* positions and aromatic N-acylated amines, e.g. acetanilide, do not react [1].

The color hues produced in the reaction do not appear to be affected by differing substituents at the amine nitrogen; however electron-attracting substituents at the α -C atom appear to reduce the detection sensitivity of the reaction [1]. The colors produced remain stable for months in the dark. In the light the zones produced by primary amines fade more rapidly than those from secondary amines [1].

The detection limits for analeptics and stimulants are 2 to 5 μ g substance per chromatogram zone [6]. β -Blockers can be detected at 50–100 ng per chromatogram zone [5].

The reagent can be employed, for instance, on silica gel, kieselguhr, Si 50000 and RP layers.

Procedure Tested

Tetracyclines [7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates RP 18 WF _{254s} (MERCK).
Mobile phase	Oxalic acid (c = 0.5 mol/L, aqueous) – acetone – methanol (27+10+6).

Migration distance 8 cm

Running time 60 min

Detection and result: The chromatogram was dried for 15 min in a stream of warm air and first examined under UV light. Tetracycline, chlorotetracycline, doxycycline and oxytetracycline fluoresced red under long-wavelength UV light ($\lambda = 365$ nm). These four substances appear as dark zones on a pale blue fluorescent background (fluorescence quenching) under short-wavelength UV light ($\lambda = 254$ nm).

The chromatogram was then immersed in dipping solution I for 1 s, dried briefly in a stream of warm air and then immersed in dipping solution II for 1 s. It was then dried in a stream of warm air for 10 min.

Tetracycline (hR_f 35–40) produced blue and doxycycline (hR_f 15–20), chlorotetracycline (hR_f 25–30) and oxytetracycline (hR_f 40–45) produced violet chromatogram zones on a yellow background. The detection limits for all 4 compounds were 2 ng substance per chromatogram zone ($\lambda = 550$ nm).

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 550$ nm (Fig. 1A) or $\lambda = 580$ nm (Fig. 1B).

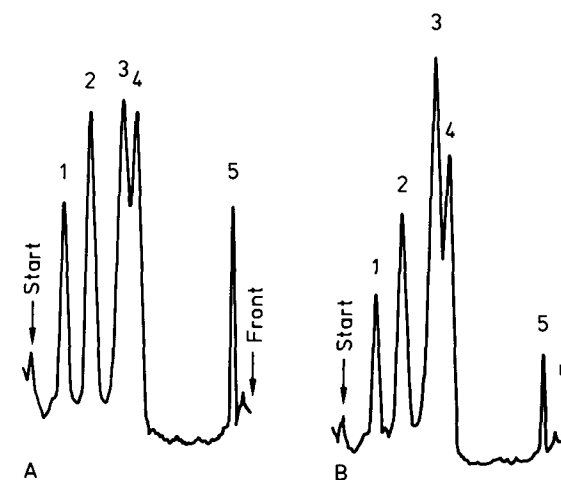


Fig 1: Reflectance scan of a chromatogram track with 16 ng of each substance per chromatogram zone: measurement at $\lambda = 550$ nm (A) and at $\lambda = 580$ nm (B). Note: The ordinate for (B) has been compressed by ca. 50% in comparison to (A): 1 = doxycycline, 2 = chlorotetracycline, 3 = tetracycline, 4 = oxytetracycline, 5 = unknown.

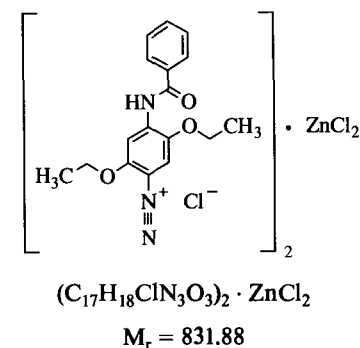
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Fast Blue Salt BB Reagent

Reagent for:

- Phenols [1]
e.g. tetracycline antibiotics [2, 3]

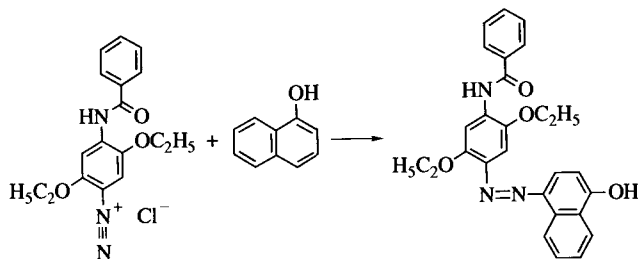


Preparation of the Reagent

Dipping solution	Dissolve 0.5 g fast blue salt BB (C.I. 37175) in 25 ml water and make up to 100 ml with methanol [1].
Spray solution	Dissolve 0.5 g fast blue salt BB in 100 ml water [2].
Storage	The reagent solutions should always be prepared fresh and fast blue salt BB should be stored in the refrigerator.
Substances	Fast blue salt BB Methanol

Reaction

Fast blue salt BB couples with phenols, preferably in alkaline medium, to yield intensely colored azo dyes.



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 4 s or homogeneously sprayed with the spray solution and then heated to 110–120 °C for 5–10 min [1, 2].

Chromatogram zones of various colors are produced on a pale yellow-orange colored background [1, 2].

Note: The dipping solution can also be used as a spray solution. RP layers should be treated with the methanol-containing reagent on account of its better wetting properties. In addition it is necessary, particularly after the use of acidic mobile phases, to spray with alkalis, e.g. pyridine, after the heat treatment step [2, 3].

The detection limits per chromatogram zone are 30–50 ng for tetracyclines [2, 3] and 2 ng for 1- and 2-naphthol [1].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000, and RP layers.

Procedure Tested

1-Naphthol and 2-Naphthol [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that have been precleaned by developing once to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.
Mobile phase	Toluene – triethylamine (30+10).
Migration distance	6 cm
Running time	12 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, cooled to room temperature, immersed in the dipping solution for 4 s and then heated to 110 °C for 5 min.

2-Naphthol (*hR_f* 50–55) appeared as a pink and 1-naphthol (*hR_f* 60–65) as a violet-green chromatogram zone on a pale yellow background. The detection limits were 2 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the absorption maximum of 1-naphthol ($\lambda_{\text{max}} = 460$ nm, Fig. 1A) or at the absorption maximum of 2-naphthol ($\lambda_{\text{max}} = 520$ nm, Fig. 1B), as required.

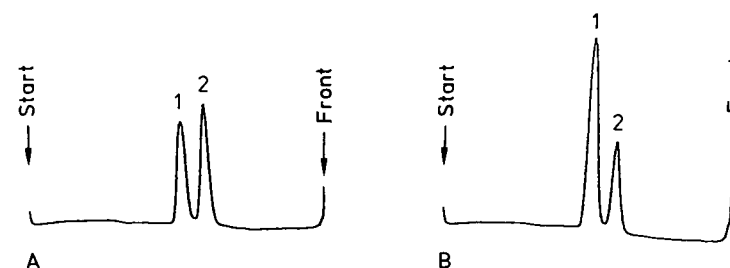


Fig. 1: Absorbance scan of a chromatogram track with 100 ng each of 2-naphthol (1) and 1-naphthol (2) per chromatogram zone: (A) scanned at $\lambda = 460$ nm, (B) scanned at $\lambda = 520$ nm.

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Iodine Reagents

It is very often advantageous in thin-layer chromatography to be able to obtain preliminary impression of a substance separation by first exposing the plate to a rapidl carried out, economically priced universal reaction before passing on to final characteri zation using group-specific or even better substance-specific reactions.

Iodine is such a universal reagent. It was introduced by MANGOLD [1] as early as 196 for the analysis of lipids and used again within a year by BARRETT [2] as a “nondestructive reagent”.

Detection by iodine is usually based on physical concentration of iodine molecule in the lipophilic chromatogram zones without any reaction occurring. Iodine is mor strongly enriched in the substance zones than in the neighboring polar, substance-free silica gel or alumina layer. The result is brown chromatogram zones on a yellow background [3].

Iodine is a less suitable reagent for use on moderately polar phases and RP material. The chemical modification of the silica gel that such layers have undergone makes ther considerably more lipophilic, so that the contrast between substance-coated chromatogram zone and substance-free background is not very strong. The same applies t polyamide layers.

Documentation is carried out as soon as the iodine-colored chromatogram zone can be readily recognized. Then the adsorbed iodine can be allowed to evaporate in th fume cupboard or vacuum desiccator, so that the same chromatograms can be subjected to further reactions and separation steps (e. g. SRS techniques, 2-D separation; coupling techniques such as TLC/GC etc.). The chromatogram zones can also b stabilized by spraying with 0.5 to 1 percent starch solution [4, 5]; the well-known blue clathrates that are formed (starch-iodine inclusion compounds) remain stable for months.

Some substances, e. g. penicillin and pyrazolinone derivatives, are poorly detected b “iodine staining” with detection limits of 2–4 µg substance per chromatogram zone [6, 7]. The limits for lipids and for opium alkaloids lie with 50–500 ng [8] in the middle nanogram range [9].

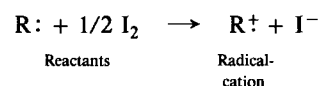
Exposure of rhodamine 6G-impregnated silica gel layers to iodine vapor for two to five minutes followed by irradiation with UV light leads to the sensitive blue coloration of the chromatogram zones on a greenish fluorescent background [8, 10].

In addition to the “iodine staining” resulting from adsorption or purely physical “solution” of the iodine molecules in the lipophilic chromatogram zones, many substances can also be made visible by chemical reaction with the iodine [9]. In such

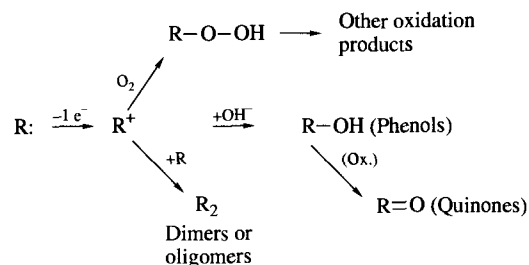
cases the interaction is not reversible since the iodine is covalently bound. It can readily be established whether there has been a chemical reaction between iodine and the test substances by application of the SRS technique [11].

Thus, in spite of its lack of reactivity, iodine reacts chemically with unsaturated compounds, whereby the silica gel of the TLC layer can sometimes be assigned a catalytic role [11, 12]. Irreversible oxidations and electrophilic substitution and addition reactions have been observed on the interaction of iodine with tertiary nitrogen compounds; such reactions possibly depend on particular steric relationships or are favored by particular functional groups [13, 14].

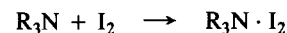
The "iodine reaction" is possibly a one-electron oxidation with the initial formation of a radical cation:



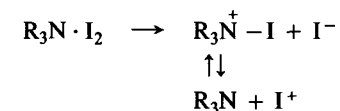
This can then react in various ways. The following products can be derived schematically:



Charge transfer complexes can also be formed, as shown using a tertiary nitrogen compound as an example. An iodine molecule first adds to the nitrogen compound:



The complex that is formed can dissociate to form a cation (*n*-σ-complex) and an iodide anion, with the iodide ion reacting with the excess iodine molecules that are present. In addition the decomposition of the *n*-σ-complex can lead to the formation of highly reactive iodine cations, which can initiate further reactions – e.g. oxidations or electrophilic substitutions of aromatic systems [11, 13].



In acidic media the *n*-σ-complex can also produce periodide anions or periodide complexes; these – like the iodide anion – are appreciably less reactive than the iodine cation [13].

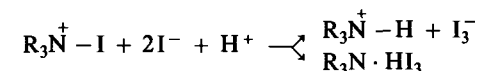


Table 1 lists examples of the observation and demonstration of such reactions.

Table 1: Examples of treatment with iodine leading to oxidation, addition or substitution products.

Substances	Sorbent	Remarks	References
Estrone derivatives	Silica gel GF ₂₅₄	Iodination to 2-iodoestrone and 2,4-diiodoestrone	[12]
Polycyclic aromatic hydrocarbons, indole and quinoline derivatives, naphthylamines, azulenes	Silica gel G	Formation of oxidation products via the initially formed iodine complexes	[15]
Polycyclic aromatic hydrocarbons	Silica gel G	Monovalent oxidation of the iodine complexes via radical cations yields dimeric or tetrameric aromatics	[16]
Pyridine, pyrrole, indole, quinoline and isoquinoline alkaloids	Silica gel G	Detection by two-dimensional TLC	[11]
Emetine, cephaeline	Silica gel 60	After iodine treatment emetine fluoresces yellow and cephaeline blue	[17]

Table 1: (continued)

Substances	Sorbent	Remarks	References
Pharmaceuticals	Silica gel	Irreversible reaction of iodine with acetylsalicylic acid, aethaverine, amidopyrine, ascorbic acid, benzocaine, quinine, dihydrocodeine, fluorescein, glycine, hydrocortisone acetate, isoniazid, metamizole, papaverine, paracetamol, phenacetin, phenol-phthalein, piperazine, resorcinol, salicylic acid, salicylamide, sulfaguanidine, thymol, triethanolamine, tris buffer; detection by reaction chromatography	[18]
Quinine alkaloids (quinine, cinchonine), barbiturate derivatives, retinol, calciferol and cholecalciferol	Aluminium oxide pH 8.6	Elimination of vinyl groups, addition of iodine to double bonds, ester cleavage; detection by IR	[14]
Morphine, oxymorphone	Silica gel	Iodination	[19]
Opium alkaloids (morphine, codeine), acetylmorphine, oxycodone, brucine, phenylbutazone, ketazone, trimethazone	Aluminium oxide pH 8.6	Iodine addition to the tertiary nitrogen of the opium alkaloids and to the OCH ₃ group of the brucine with formation of an <i>o</i> -quinone derivative, probably ring opening in the case of phenylbutazone, ketazone and trimethazone; detection by IR	[13]
Thiols and thioethers (dithiaden, prothiaden, thiamine etc.)	Aluminium oxide pH 8.3	Oxidation of sulfur and attack of the double bond in the thiazole ring	[20]
Alkaloids (codeine, brucine), phenothiazines (promethazine), sulfonamides (sulfathiazole), vitamins (axerophthol, cholecalciferol)	Aluminium oxide pH 8.6	Irreversible complex formation or saturation of double bonds; detection by IR	[21]

So a check must always be made of whether the universal iodine reagent can be used for nondestructive testing or whether the substances undergo irreversible changes. The iodine reagents are preferentially used for the detection of lipophilic substances (fats, waxes, PAH's etc.) and since this chapter would be inordinately long if all the substances were listed and the references cited the iodine monographs that follow only include those classes of compounds where the use of iodine as a detection reagent seems unusual.

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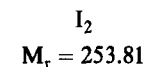
Iodine Vapor Reagent

Reagent for :

- Lipids
 - e.g. fats, waxes, hydrocarbons [1-4]
 - free fatty acids, diglycerides [5], fatty acid esters [6],
 - prostaglandins [7], arene-cyclopentadienyl iron complexes [8]
- Phospholipids
 - e.g. lecithin [5, 9-11], sphingomyelin [10]
 - phosphatidylcholine [12], diolelphosphatidylcholine [13]
 - ceramides [14], gangliosides [15]
- Steroids [16, 17]
 - e.g. cortisone, testosterone, corticosterone [18]
 - estrone [19], 19-norsteroids [20]
- Carotenoids [21]
- Antioxidants
 - e.g. cyclopentylphenols [22]
- Hydroxyacetophenone [23] and benzophenone derivatives [24]
- N-containing glycolate esters [25]
- Diethyl phenyl phosphate derivatives [26]
- Detergents and emulsifiers
 - e.g. dodecyl benzenesulfonate, Triton X-100 [27]
 - alcohol ethoxilates [27, 28],
 - arlacel A [29]
- Polymers
 - e.g. polyethylene glycol derivatives [30, 31]
 - polystyrene, polytetrahydrofuran [31]
- Pesticides
 - e.g. carbamates [32]
 - such as propamocarb [33]
 - e.g. aziridine derivatives [34]
- Purine derivatives
 - e.g. theophylline, caffeine [35, 36]

Reagent for :

- Pharmaceuticals
 - e.g. antibiotics
 - such as penicillins [37], maridomycines [38], ferrithiocin [39]
 - e.g. amphetamines
 - such as phentermine, methamphetamine, ephedrine [40]
 - N-cyanobenzylamphetamine [41]
 - e.g. benzodiazepines
 - such as chlordiazepoxide [35], diazepam [35, 36]
 - e.g. miscellaneous pharmaceuticals [42]
 - such as propoxyphene [43], barbital [36], phenacetin, antipyrin [35],
 - triamcinolone acetonide ester [44]
 - germine, gergmine acetate and diacetate [45],
 - xanthone [46], opium alkaloids [47], captopril [45]
- Mycotoxins
 - e.g. slaframine [48]
- Carbohydrates [49]



Preparation of the Reagent

Iodine reagent	Place a few iodine cystals on the base of a chamber that can be tightly sealed (e.g. twin- trough chamber). Violet iodine vaporizes and distributes itself homogeneously throughout the interior of the chamber after a few hours. Gentle warming of the iodine chamber accelerates the vaporization of the iodine.
Starch dipping solution	Dissolve 0.5 g soluble starch in 100 ml water with heating.
Storage	The iodine chamber should be stored in the fume cupboard.
Substances	Iodine Starch, soluble

Reaction

Iodine is enriched to a greater extent in chromatogram zones coated with lipophilic substances than it is in a hydrophilic environment. Hence, iodine is only physically “dissolved” or adsorbed. Occasionally a chemical reaction also takes place, such as, for example, with estrone [19] (cf. “Iodine Reagents”). In general it may be said that the longer the iodine effect lasts the more oxidations, additions or electrophilic substitutions are to be expected.

Method

The chromatograms are freed from mobile phase in a stream of warm air, cautiously placed in the iodine chamber and left there for varying periods of time, depending on the substance class (e.g. 5–10 s [1], 15–30 s [17, 50], 1 min [38], 5–15 min [11, 25] or even up to 30 min [22, 24] or for several hours [42].

Brown-violet chromatogram zones are generally formed on a yellow background and, in some cases, these fluoresce when viewed under UV light ($\lambda = 254$ or 365 nm) after the evaporation of the excess iodine. Sometimes colorless chromatogram zones are formed on a brown background, for example, if the iodine reacts chemically with the substances that are chromatographed.

Carotenoids immediately form an olive-green complex, which fades irreversibly if the exposure to iodine is prolonged [21].

Note: The iodine evaporates relatively quickly from the layer after a chromatogram has been removed from the iodine chamber. This evaporation of the iodine can be considerably delayed by covering the chromatogram with a glass plate; the edge can also be sealed with adhesive tape, if necessary. Iodine solution can also be used for detection instead of iodine vapor.

Since iodine possesses fluorescence-quenching properties (true fluorescence quencher) iodine-containing chromatogram zones on layers containing fluorescence indicator F_{254} appear as dark zones on a yellow-green fluorescent background when viewed under UV light ($\lambda = 254$ nm) — even if there are only traces of iodine in the chromatogram zones.

The chromatogram zones colored by iodine can be fixed later by treatment with a 0.5–1% aqueous starch (amylose) solution. This yields the well known, deep blue iodine-starch inclusion complex which is stable over a prolonged period. This reaction

is very sensitive and, hence, should only be carried out if there are only traces of iodine in the chromatogram zones, otherwise the whole background will be colored blue.

Occasionally the starch treatment yields white chromatogram zones on a blue background (cf. procedure tested 2). This is probably a result of iodine being consumed by a chemical reaction with the zone leaving a large quantity of iodine in the background for the formation of a starch-iodine complex. Such effects are not observed on cellulose, CN and water-wetted RP 18 layers, possibly because insufficient adsorbed iodine is available in the neighborhood of the chromatogram zones for the formation of the blue complex or because, for example, the CN phase is not homogeneously wetted by the starch solution.

The detection limits are generally a few μg substance per chromatogram zone. However, the iodine detection is appreciably more sensitive for some substances: it is possible to detect 200 ng glucose [49] and 10 ng propamocarb per chromatogram zone [33].

The reagent can be used most advantageously on aluminium oxide, silica gel, kieselguhr, Si 50000, cellulose, diol and water-wettable RP 18 layers; there is less contrast in color on strongly hydrophobic RP 18 phases. NH_2 and polyamide layers are not suitable because the iodine is too strongly bound and the whole layer is colored green-yellow.

Procedure Tested 1

Fatty Oils [51]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates cellulose (MERCK) that have been impregnated by dipping in a solution of liquid paraffin — petroleum ether (5+95) ($40\text{--}60^\circ\text{C}$) for 4 s and then dried in the air for 5 min, before application of the samples.
Mobile phase	Acetic acid (99%)
Migration distance	12 cm
Running time	1.5 h

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed in an iodine chamber for 5 min. Brown chromatogram zones were

formed on a light brown background (Fig. 1A). After waiting for a few minutes while the excess iodine evaporated from the layer the chromatogram was immersed in the starch solution for 1 s and dried in a stream of warm air. It was then possible to recognize the oil components as blue chromatogram zones on a light background (Fig. 1B).

In situ quantitation: The reagent was not suitable for quantitative in situ evaluations.

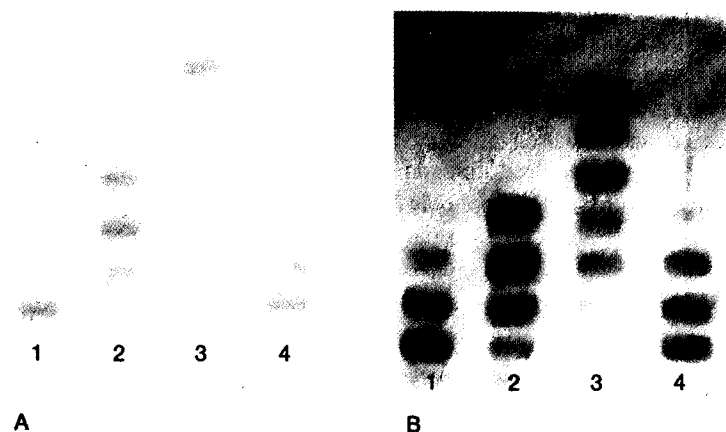


Fig. 1: Chromatogram of fatty oils (9 μ g each per 10 mm band) after iodine vapor treatment (A) and after additional immersion in a starch solution (B): Track 1: avocado oil, Track 2: sunflower oil, Track 3: linseed oil, Track 4: almond oil.

Procedure Tested 2

Detergent Dehydrol LS 3 [51]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 with concentrating zone (MERCK).
Mobile phase	1-Propanol – glacial acetic acid (90 + 10).

Migration distance 10 cm

Running time 1.75 h

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed in an iodine chamber for 20 min. Brown chromatogram zones were formed on a pale yellow background (Fig. 2A); these faded very rapidly. Therefore, the chromatogram was immediately immersed for 1 s in the starch dipping solution and dried in a stream of cold air.

Pink-colored chromatogram zones appeared on a blue background; these rapidly changed color to white zones (Fig. 2B).

In situ quantitation: The reagent was not suitable for in situ evaluations.

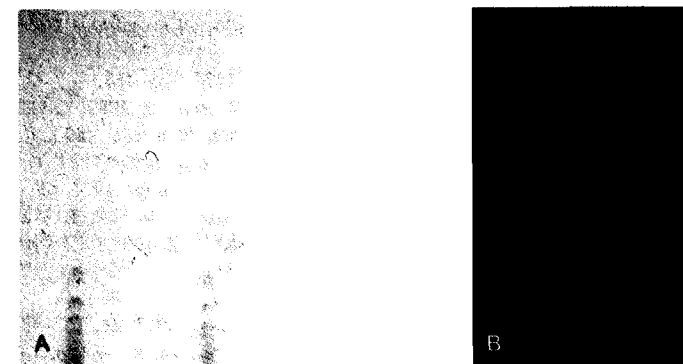


Fig. 2: Chromatogram of the detergent dehydrol LS 3 after iodine treatment (A) and after additional treatment with starch solution (B); amount applied each time 10 μ g as spots.

References

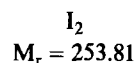
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Iodine Solution, Neutral Reagent

Reagent for:

- Lipids (fats, waxes, hydrocarbons)
 - e.g. nitrobenzylarenes [1], cholesteryl esters [2, 3], lipopurothionine [4] mono- and diglycerides [5] unsaturated lipids [6]
- Phospholipids
 - e.g. phosphatidylcholine [7], phosphatidic acid [5] phosphonolipids [8, 9]
- Polymers
 - e.g. polyisoprene, polybutadiene [10] poly(methylmethacrylate) [10, 11] polyethylene glycols [12]
- Pyrimidine nucleoside derivatives
 - e.g. uridine derivatives [13]
- Alkaloids
 - e.g. codeine [14], emetine, cephaeline [15, 16] psychotrine [16], hippadine [17]
- Pharmaceuticals [18]
 - e.g. benzodiazepines [19], thalidomide [20] dithiocarbamoylhydrazine [21], neostigmine [14] 4-amidinophenylpyruvic acid [22] 6-mercaptopurine derivatives [23] cinicene, artemisiifolin, salonitenolide [24]
- Tertiary amines and quaternary ammonium compounds [25–27]
- Pyrrolidine derivatives [28]
- Imidazole derivatives [29]
- Pentoxifylline [30]



Preparation of the Reagent

Dipping solution	Dissolve 250 mg iodine in 100 ml petroleum ether [31].
Spray solution	Dissolve 1 to 5 g iodine in 100 ml methanol [2, 3, 5–7, 11, 12], chloroform [1, 4, 16, 18, 20, 24], carbon tetrachloride [15, 28], petroleum ether [17], diethyl ether [26] or acetone [30].
Storage	The reagent solutions may be stored in the refrigerator for an extended period.
Substances	Iodine Methanol Chloroform Carbon tetrachloride Petroleum ether Diethyl ether Acetone

Reaction

Iodine is enriched to a greater extent in chromatogram zones with a coating lipophilic substances than it is in a hydrophilic environment. Hence iodine is physically “dissolved” or adsorbed. A chemical reaction occasionally takes place (cf. “Iodine Reagents”).

Method

The chromatograms are freed from mobile phase in a stream of warm air, immerse in the dipping solution for 3 s or homogeneously sprayed with the spray solution. On exceptional cases, such as, for example, in the detection of emetine and cephaeline the chromatograms then heated to 60–80 °C for 10–20 min [15, 16, 31].

Brown chromatogram zones are generally produced on an almost colorless to beige background; the zones occasionally fluoresce when examined under UV light ($\lambda = 254$ or 365 nm) [15, 16].

Note: The last traces of mobile phase must be removed completely — if necessary by heating the chromatogram — before the reagent is applied [10]. For documentation it is recommended that the chromatogram treated with iodine be covered with a glass plate in order to prevent evaporation of the iodine.

An aqueous 1% starch (amylose) spray can be sprayed on later to intensify the color contrast between the chromatogram zones and the layer background; the well known blue-colored iodine starch inclusion complexes are formed. This later treatment with starch solution should only be carried out when the iodine excess has evaporated from the layer background so that only traces of iodine remain in the chromatogram zones. Otherwise the whole chromatogram will be colored dark blue (test at a corner of the chromatogram!).

Iodine vapour can be employed as reagent in place of the iodine solution. Water-resistant layers can be treated with 0.5 to 1% aqueous iodine solutions, e.g. 1 g iodine and 2 g potassium iodide dissolved in 30 ml water and made up to 100 ml with ethanol or LUGOL's solution or dilute iodine tincture. These solutions all contain in addition potassium iodide to form the stable complex $KI \cdot I_2$ with the iodine. This greatly reduces the evaporation of the iodine from the chromatogram so that later treatment of the chromatogram with the starch solution can lead to a complete blue coloration of the whole chromatogram, even after an extended period of waiting. Such a potassium iodide-containing iodine solution has been employed for example for the detection of choline [32].

In the case of quaternary ammonium compounds the chromatograms can be treated afterwards with sodium nitrite solution in order to intensify the color [27].

The detection limits are mostly in the microgram range, for cephaeline and emetine they are 200 pg substance per chromatogram zone.

The reagent can be used, for instance, on aluminium oxide, silica gel, kieselguhr and Si 50000 as well as on cellulose, polyamide, RP, CN, Diol and NH_2 phases.

Procedure Tested

Alkaloids in *Ipecacuanha* Tincture [31]

Method	Ascending, one-dimensional, two-fold development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).

Mobile phase Dichloromethane — methanol (85+15).

Migration distance 2 x 7 cm

Running time 2 x 15 min

Detection and result: The chromatogram was dried in a stream of warm air for 5 min, immersed in the dipping solution for 3 s and then, after brief evaporation of the excess iodine, heated to 60°C on a hot plate for ca. 10 min. After cooling to room temperature it was placed for a further 5 min in the vacant trough of a twin-trough chamber, whose second chamber had been filled with 25% ammonia solution.

Cephaeline (hR_f 6–11) appeared as blue and emetine (hR_f 10–15) as yellow fluorescent chromatogram zones on a dark background when examined under long-wave-length UV light ($\lambda = 365$ nm) (Fig. 1). Emetine — like cephaeline — also yielded a blue fluorescence on RP-2 and RP-18 layers.

The detection limit was ca. 200 pg for emetine.

Note: Under the conditions employed emetine and cephaeline were not well separated but there was good resolution of the subsidiary alkaloids of the *ipecacuanha* tincture (Fig. 1). The separation and quantitative determination of the main alkaloids (Fig. 2) can be carried out under the following conditions: Ascending, one-dimensional development in a trough chamber with chamber saturation; layer: HPTLC plates Silica gel 60 (MERCK); mobile phase: dichloromethane — methanol — ammonia solution (25%) (34+6+1); migration distance: 6 cm; running time: 13 min; hR_f : cephaeline 65–70; emetine 75–80.



Fig. 1: Chromatogram of *ipecacuanha* tincture and of the standard substances: 1 = emetine, 2 = tincture, 3 = cephaeline.

In situ quantitation: For fluorimetric evaluation excitation was carried out at $\lambda_{\text{exc}} = 313 \text{ nm}$ and the fluorescence emission of emetine was measured at $\lambda_{\text{fl}} > 460 \text{ nm}$ (cut off filter Fl 46).

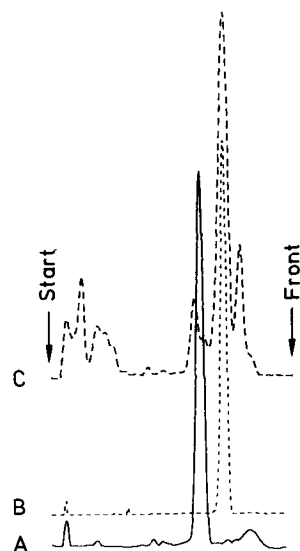


Fig. 2: Fluorescence scan of the chromatogram tracks of the standard substances cephaeline (A) and emetine (B) and of the *ipeacacuanha* extract (C). Amounts applied: cephaeline $0.5 \mu\text{g}$, emetine $0.7 \mu\text{g}$ per 10 mm track length.

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Iodine–Potassium Iodide Solution, Acidic Reagent

Reagent for:

- Alkaloids [1]
- Polyethylene glycols [1]
- Pharmaceuticals
 - e.g. tertiary nitrogen compounds
such as phenothiazines [2], phenylalkylamines,
chlorphentermine, cloforex [3]
 - e.g. antibiotics
such as ampicillin and oligomers [4]
penicillin [5]
 - e.g. purine derivatives
such as caffeine, theobromine, theophylline [6–8]
- Pesticides
 - e.g. fungicides
such as ridomil [9]



Preparation of the Reagent

Dipping solution Dissolve 0.4 g potassium iodide and 0.8 g iodine in 40 ml 96% ethanol and add 10 ml 25% hydrochloric acid [8].

Spray solution I *For alkaloids:* Dissolve 1 g iodine and 10 g potassium iodide in 50 ml water with warming, add 2 ml glacial acetic acid and make up to 100 ml with water [1].

Spray solution II *For purine derivatives:*
Solution A: Dissolve 0.1 g potassium iodide and 0.2 g iodine in 10 ml 96% ethanol [6, 7].
Solution B: Mix 5 ml 25% hydrochloric acid with 5 ml 96% ethanol [6, 7].

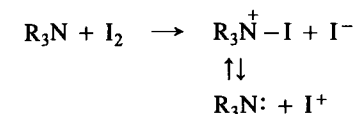
Spray solution III *For antibiotics:*
 Mix 100 ml 1% starch solution with 8 ml acetic acid and 1 ml 0.1 N iodine solution [4].

Storage The reagent solutions may be stored for an extended period.

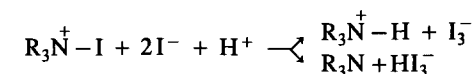
Substances
 Iodine
 Potassium iodide
 Hydrochloric acid (25%)
 Ethanol (96%)
 Acetic acid (100%)
 Starch, soluble

Reaction

Tertiary nitrogen and iodine initially form a n - σ -complex, from which a strongly reactive iodine cation is produced; this cation can bring about electrophilic substitutions on aromatic systems or cause oxidations [2].



In acidic medium the n - σ -complex can also form periodide anions or periodide complexes and these — like the iodide anion — are appreciably less reactive than an iodine cation [2].



The detection of antibiotics depends on the fact that the iodine contained in the reagent reacts chemically with these and, hence, is no longer available in the chroma-

togram zones for the formation of the deep blue-colored iodine-starch inclusion compound. In the case of penicillin derivatives the β -lactam ring is initially opened by alkali treatment or with suitable enzymes [10, 11]. The penicilloic acid thus formed reacts rapidly consuming 9 equivalents of iodine [10].

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s or homogeneously sprayed with the suitable spray solution and then dried in air or in a stream of cold air. Alternatively purine derivatives can be treated successively with the two spray solutions IIA and IIB (with 2 min air drying in between) [6, 7].

Spray solution III, which is suitable for the detection of antibiotics, also contains potassium iodide! To detect penicillin derivatives the chromatograms must first be placed – while still damp with mobile phase – for ca. 15 min in an ammonia chamber before treatment with spray solution III [5].

This generally produces red-brown to blue-violet chromatogram zones on a sand-colored background. The detection of antibiotics with spray solution III yields almost colorless chromatogram zones on a blue background [4, 5].

Note: The dipping solution can also be used as spray solution. Since the chromatogram zones slowly fade in the air it is recommended that the chromatograms be covered with a glass plate for long-term storage. Color differentiation is possible with purine derivatives [6, 8]. Diprophylline is not colored [6].

The subsequent treatment with starch solution frequently employed after the iodine treatment for the stabilization and enhancement of the „iodine“ chromatogram zones cannot be employed here since the layers – even after lying in the air for several hours (evaporation of the excess iodine) – still contain so much iodine that the whole background is colored blue [8].

The detection limits for purine derivatives are 120–400 ng and for antibiotics 50 ng substance per chromatogram zone [4].

The reagent can, for instance, be employed on silica gel, kieselguhr, Si 50000, cellulose and NH_2 layers; the reaction is appreciably less sensitive on RP 18, CN and Diol phases, neither is there any color differentiation of the purine derivatives [8]. Cellulose and polyamide layers are not suitable, since the whole layer background is colored dark brown [8].

Procedure Tested

Purine Derivatives [6, 8]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Acetone – ethyl acetate – ammonia solution (25%) (75 + 23 + 2).
Migration distance	6 cm
Running time	15 min

Detection and result: The chromatogram was dried in a stream of warm air, immersed in the dipping solution for 2 s, dried in the air for 2 min and then dried for a further 2 min in a stream of cold air. Theophylline (hR_f 15–20) and caffeine (hR_f 65–70) were immediately visible as blue-violet chromatogram zones, while etophylline (hR_f 40–45) and proxiphylline (hR_f 60–65) appeared a little later as brown zones on a sand-colored background (Fig. 1A). The detection limits for theophylline and caffeine lay at 120 ng substance and for etophylline and proxiphylline at 400 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric scans were carried out in reflectance at a wavelength of $\lambda = 500 \text{ nm}$ (Fig. 1B).



Fig. 1: A) Chromatogram of xanthine derivatives: Track 1: theophylline, Track 2: proxiphylline, Track 3: mixture, Track 4: etophylline, Track 5: caffeine. B) Reflectance scan of a chromatogram track with 1.7 μg theophylline (1), 3 μg each etophylline (2) and proxiphylline (3) and 1.6 μg caffeine (4) per chromatogram zone.

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Iodine-Potassium Iodide Solution–Sodium Azide–Starch Reagent (Awe's Reagent, Iodine Azide Reaction)

Reagent for:

- Sulfur-containing compounds [1–3]
 - e.g. thiols [4, 5] and thioethers [5]
 - sulfide ions [4]
 - thiourea derivatives
 - such as phenylethyl- and 4-pentenylthiourea [4]
 - N-ethyl-N'-benzylthiourea [6]
 - oxazolidinethione derivatives [4]
 - thiazolidine derivatives [7]
 - S-containing amino acids
 - such as cystine, methionine [3], cysteine [8]
 - thiohydantoin derivatives [17], PTH amino acids [9–12]
- Penicillin derivatives and cephalosporins
 - e.g. penicillic acid, oxacillin, clonacillin, methicillin, ampicillin, cephonin, cephalosporin C [8]
- Thiophosphorus compounds
 - e.g. phosphordithioates, disulfides, alkylphosphinsulfides [5]
- Pesticides
 - e.g. thiophosphoric acid insecticides [5]
 - 1,4-oxathiine derivatives [13]
 - ridomil (acylon) [14]

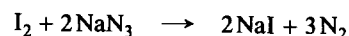
I_2	NaN_3	$(\text{C}_6\text{H}_{10}\text{O}_5)_n$
$M_r = 253.81$	$M_r = 65.01$	$M_r = 6500-8000$
Iodine	Sodium azide	Starch

Preparation of the Reagent

Dipping solution I	Dissolve 0.5 g soluble starch in 100 ml water by heating to boiling [15].
Dipping solution II	Dissolve 0.25 g iodine and 0.4 g potassium iodide in 100 ml water. Dissolve 0.4 g sodium azide in this solution [15].
Spray solution II	Dissolve 1–3 g sodium azide in 100 ml 0.005 N [8] to 0.1 N iodine solution [1, 13] and dilute with 100 ml ethanol, if necessary [6]. Alternatively, a solution of 1.27 g iodine in 100 ml 95% ethanol can be mixed with a solution of 3.25 g sodium azide in 75% ethanol in the volume ratio 1+1, immediately before spraying [14].
Storage	Dipping solution I may be stored for an extended period; dipping solution and spray solution II should be stored in the refrigerator and made up fresh every other day [11].
Substances	Iodine Sodium azide Potassium iodide Iodine solution 0.05 mol I ₂ /L (= 0.1 N sol'n) Starch, soluble Ethanol

Reaction

The detection depends on the “iodine azide reaction” that normally takes place very slowly and during the course of which sodium azide reacts with iodine to form sodium iodide with the production of nitrogen:



This reaction is catalyzed by the presence of divalent sulfur – e.g. sulfur ions, substances with S-S links, thioethers, thiazoles. If such substances are present in a chromatogram zone then the iodine applied with the reagent is consumed by reaction with sodium azide according to the above reaction. It is no longer available for the for-

mation of the deep blue-colored iodine starch inclusion compound (clathrate complex) with the starch solution that is also applied [3, 13, 16].

Method

The chromatograms are freed from mobile phase in a stream of warm air, initially immersed in dipping solution I for 1 s or sprayed homogeneously with it and then, after brief intermediate drying in a stream of cold air, either immersed in dipping solution II for 1 s or homogeneously sprayed with spray solution II.

In the case of penicillins and cephalosporins the chromatograms are first sprayed with 2 N sodium hydroxide solution [8].

This yields colorless to slightly yellow chromatogram zones on a deep blue-colored background.

Note: Alternatively 1% solutions of starch, iodine and sodium azide may be sprayed successively onto the chromatogram in that order [4, 9]. Other orders of application are also referred to in the literature [1, 2, 17] and sometimes the starch is also worked into the layer so that it is not necessary to spray with it [11, 12]. Sometimes the treatment of the chromatograms with starch solution is omitted [5, 6, 14]; in such cases colorless chromatogram zones appear on a brown layer background.

However, the starch solution should not be omitted completely since the color difference between the chromatogram zones, in which the iodine is reduced to colorless iodide according to the “iodine azide reaction” mentioned above, and the background colored brown by unreacted iodine is considerably less than the difference in color between the deep blue background provided by the starch-iodine clathrate complex and the pale chromatogram zones.

The blue coloration of the background rapidly changes to brown [11] and then gradually fades [8]. The color change of the background from blue to brown on complete drying of the layer is reversible: The blue color can be regenerated by treatment with water vapor [15].

Ridomil, with which the treatment with starch solution was not carried out, is said to yield brown chromatogram zones on a pale yellow background [14]. Hence, it may be assumed that this detection is based not on the “iodine azide reaction” but on the physical adsorption and enrichment of iodine in the lipophilic chromatogram zones (cf. “Iodine Reagents”).

The detection limits for ridomil are 2.5 µg, for penicillin derivatives and cephalosporins 0.5–1 µg [8], for thiophosphate compounds 30 ng – 10 µg and for PTH amino acids and 1,4-oxathiine derivatives 200 ng substance per chromatogram zone [11, 13].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, NH₂ and Diol layers; RP-18W, CN, polyamide and cellulose phases are not suitable [15].

Procedure Tested

Thiophosphate Insecticides [15]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	<i>n</i> -Hexane – tetrahydrofuran (5+1).
Migration distance	7 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in dipping solution I for 1 s and then dried for 5–10 min in a stream of warm air. Then the cooled TLC plate was immersed for 1 s in dipping solution II.

Azinphos ethyl (*hR_f* 20–25), malathion (*hR_f* 40–45) and diazinone (*hR_f* 47–52) yielded white chromatogram zones on a blue background immediately. Before in situ quantitation the chromatogram was dried in the air until no film of moisture could be seen on the layer surface. It was then dried completely in a stream of warm air whereby the blue coloration of the background changed to brown (Fig. 1). The visual detection limits were 200 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric measurements in reflectance were made at a wavelength $\lambda = 590$ nm (Fig. 2).

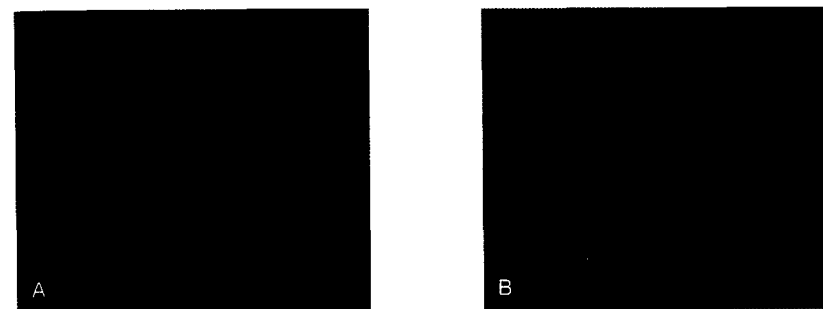


Fig. 1: Chromatogram of the thiophosphate insecticides (each ca. 500 ng) after treatment with dipping solutions I and II (A) before and (B) after complete drying of the TLC plate. Tracks 1 and 5: mixture; Track 2: azinphos ethyl; Track 3: malathion; Track 4: diazinone.

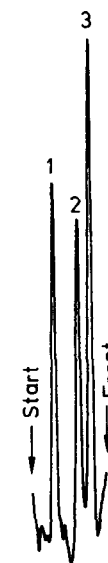


Fig. 2: Reflectance scan of a chromatogram track with 500 ng azinphos ethyl (1), 580 ng malathion (2) and 590 ng diazinone (3) per chromatogram zone.

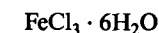
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Iron(III) Chloride Reagent

Reagent for:

- Phenols [1-7]
 - e.g. flavonoid glycosides and aglycones [8-14]
 - catechols [15, 16]
 - tannins [17-19]
 - such as gallic acid and its esters [16]
 - phenolic pharmaceuticals [20]
 - such as phenothiazines [20]
 - acetylsalicylic acid [22]
 - hydroxycinnamic acid derivatives
 - such as caffeic acid, chlorogenic acid [23]
 - 1-hydroxyacridone alkaloids [24]
 - gentisyl alcohol [25], gentisyl acid [20]
 - fungal toxins
 - such as orellanin, orellinin, orellin [26]
 - mycophenolic acid [27]
- Enols
 - e.g. α -ketolactones [28]
- Hydroxamic acids [7, 29]
- Mycotoxins
 - e.g. penitrem A [30]
- Cholesterol and its esters [34]
- Ergot alkaloids [35]
- Inorganic anions
 - e.g. nitrite, iodate, chromate, vanadate, selenite, selenate, hexacyanoferrate(II) and (III) ions [31]
 - thiocyanate (rhodanide) ions [32]



$$M_r = 270.30$$

Preparation of the Reagent

Dipping solution	Dissolve 1 g iron(III) chloride hexahydrate in 5 ml water and make up to 100 ml with ethanol [7].
Spray solution	Dissolve 0.1 to 10 g iron(III) chloride hexahydrate in 100 ml ethanol [9, 17, 19, 21, 24], methanol [3, 4, 8, 15, 23, 27], 1-butanol [30], water [5, 20, 31, 32] or dilute hydrochloric acid [7, 29].
Storage	The reagent solutions may be stored in the refrigerator (4 °C) for several days.
Substances	Iron(III) chloride, hexahydrate Methanol Ethanol (95 %)

Reaction

Iron(III) chloride forms colored complexes with phenols.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and then heated to 100–110 °C for 5–10 min.

Variously colored chromatogram zones are produced, usually before heating, on a colorless to pale beige-yellow background.

Flavanones appear red to blue-violet [8], other flavonoid glycosides green, red-brown, wine red and red to blue-violet [11, 13], catechols green to blue [15], tannins blue [17], phenothiazines pink [21], 1-hydroxyacridone alkaloids green [24], penitrem A green to blue-green [30], inorganic anions pale yellow to blue-green [31].

Note: Dilute acetic acid [22], conc. sulfuric acid [25, 34, 35] have also been recommended for making up the reagent, as have anhydrous iron(III) chloride in dioxane or

chloroform [5]. A post-reaction treatment with saturated sodium carbonate solution was described in some cases [23].

The detection limits for phenols are 20–100 ng substance per chromatogram zone [33].

The reagent can, for instance, be employed on silica gel, kieselguhr, Si 50000, cellulose and polyamide layers.

Procedure Tested

Aminophenols [33]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that have been precleaned by developing once to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.
Mobile phase	Toluene – methanol (18+9).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air for 10 min, immersed in the dipping solution for 1 s and then heated to 110 °C for 5 min on a hotplate.

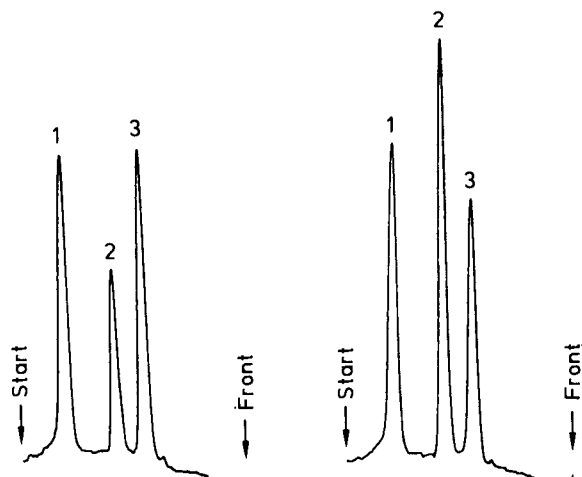
On a beige-yellow background 2-aminophenol (hR_f 55–60) appeared as ochre-brown, 4-aminophenol (hR_f 40–45) as violet-brown and 4-aminosalicylic acid (hR_f 20–25) as pale brown-violet chromatogram zones.

The detection limits lay at 20 ng substance for 2-aminophenol, 50 ng substance for 4-aminosalicylic acid and 100 ng substance for 4-aminophenol per chromatogram zone.

In situ quantitation: The absorption photometric scans were carried out in reflectance at a wavelength of either $\lambda = 460$ nm (λ_{\max} (2-aminophenol)) or $\lambda = 520$ nm (λ_{\max} (4-aminophenol)) (Fig. 1).

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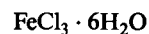


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Iron(III) Chloride–Potassium Hexacyanoferrate(III) Reagent (Barton's Reagent)

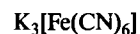
Reagent for:

- Aromatic amines [1, 3]
e.g. anilines
- Phenols [3]
e.g. salsonilol, dopamine [4],
lignans, pyrogallol derivatives [5],
zingerone [7],
gallic acid, gallotannins [8],
curare alkaloids (tubocurarine [9])
- Phenolic steroids [10–13],
e.g. estrone, estradiol, estriol
- Analgesics
e.g. aminophenazone [14]
- Anti-inflammatories
e.g. carprofen, zomepirac, diclofenac [15]
- Enamino ketones [16]
- Enol ketones [17]
- Thiosulfates [3]
- Isothiocyanates (mustard oils) [3, 19, 20]
- Thiourea derivatives [19, 20]
- Degradation products of carbamate insecticides [18]
e.g. mexacarbates, matacil, landrin



$$M_r = 270.30$$

Iron(III) chloride



$$M_r = 329.26$$

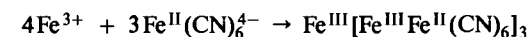
Potassium hexacyanoferrate(III)

Preparation of the Reagent

Solution I	Dissolve 1 g potassium hexacyanoferrate(III) in 100 ml water.
Solution II	Dissolve 2 g iron(III) chloride hexahydrate in 100 ml water.
Solution III	Dissolve 0.3 to 2 g potassium hexacyanoferrate(III) in 100 ml water.
Solution IV	Dissolve 0.3 to 10 g iron(III) chloride in 100 ml water.
Dipping solution	Immediately before use mix 10 ml water, 8 ml solution I, 2 ml solution II and 1 ml 32% hydrochloric acid in a measuring cylinder and make up to 100 ml with methanol [2].
Spray solution	Immediately before use solutions III and IV are mixed, generally in equal proportions [1, 3, 6–8].
Storage	Solutions I – IV may be stored in the refrigerator with the exclusion of light for ca. 2 weeks [6].
Substances	Iron(III) chloride hexahydrate Potassium hexacyanoferrate(III) Hydrochloric acid (32%) Methanol

Reaction

Some of the iron(III) ions in the reagent are converted to iron(II) ions by reducing substances and then react to produce Prussian blue or TURNBULL's blue.



Method

The chromatograms are freed from mobile phase in a stream of warm air, then immersed in the dipping solution for 2 s or uniformly sprayed with the spray solution. In the case of anti-inflammatories, the plates are then heated to 110°C for 5–10 min [15].

Blue chromatogram zones are produced on an almost colorless to pale yellow background.

Note: The dipping solution can also be used as a spray solution. When using the spray solution it is possible to increase the color intensity by spraying afterwards with hydrochloric acid ($c = 2$ mol/L) [16]. The blue chromatogram zones remain readily visible for ca. 15 to 30 min; then they begin to fade, while the background gradually acquires a blue coloration [14].

The detection limits per chromatogram zone are 100–200 ng for gallic acid and aminophenazone [8, 14] and 10–15 ng for uric acid [6].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Substituted Anilines [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Toluene
Migration distance	8 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for ca. 10 min, immersed in the dipping solution for 2 s, dried for 5 min in a stream of cold air and then heated to 110°C for 1 min.

2,3-Dimethylaniline (hR_f 5–10), 4-chloro-2-methylaniline (hR_f 15–20), 3,4-dichloroaniline (hR_f 25–30), 3,5-dichloroaniline (hR_f 40–45), 2,3-dichloroaniline (hR_f

45–50) and 2,5-dichloroaniline (hR_f 60–65) appeared as pale blue-turquoise-colored chromatogram zones on a beige-colored background. The detection limits per chromatogram zone ranged from 2 ng (2,3-dimethylaniline) to 10 ng (4-chloro-2-methylaniline).

In situ quantitation: The absorption photometric scan in reflectance was carried out at a mean wavelength of $\lambda = 670$ nm (Fig. 1).

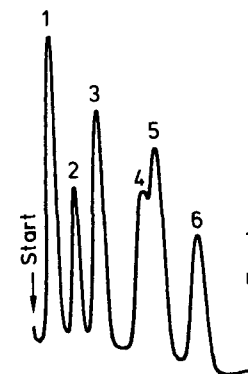


Fig. 1: Reflectance scan of a chromatogram track with 200 ng 2,3-dimethylaniline (1), 100 ng each of 4-chloro-2-methylaniline (2), 3,4-dichloroaniline (3), 3,5-dichloroaniline (4) and 200 ng each of 2,3-dichloroaniline (5) and 2,5-dichloroaniline (6) per chromatogram zone.

References

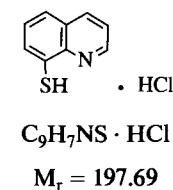
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8-Mercaptoquinoline Reagent

Reagent for:

- Cations
e.g. tin, lead, copper [1]

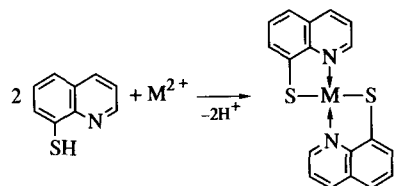


Preparation of the Reagent

Dipping solution	Dissolve 100 mg 8-mercaptoquinoline hydrochloride (thioox hydrochloride) in 100 ml ethanol [1].
Storage	The dipping solution may be kept in the refrigerator for 1 weeks [1].
Substances	Thiooxine hydrochloride Ethanol

Reaction

Like 8-hydroxyquinoline [2] 8-mercaptoquinoline forms colored and fluorescent cc plexes with many metal cations.



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 3 s or sprayed homogeneously with it and then dried in a stream of cold air for 5 min.

Yellow-colored chromatogram zones are produced that fluoresce yellow when exposed to long-wavelength UV light ($\lambda = 365$ nm).

Note: The detection limits are in the lower nanogram range.

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, cellulose and RP layers.

Procedure Tested

Organotin Compounds [1]

Method	Ascending, one-dimensional stepwise development (10 min intermediate drying in a stream of cold air) in a trough chamber with chamber saturation during the second development.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	1. Diethyl ether – glacial acetic acid (20+1). 2. Isobutyl methyl ketone – glacial acetic acid – <i>n</i> -hexane – tetrahydrofuran – dioxane (8+2+1+1+1).
Migration distance	1. 1.5 cm 2. 6 cm
Running time	1. 2 min 2. 15 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air. The organotin compounds were then converted to tin(IV) species by irradiating intense for 20 min with UV light. Then the layer was immersed in the reagent solution for 3 min and dried for 5 min in a stream of cold air.

Dimethyltin dichloride (hR_f 15–20), trimethyltin chloride (hR_f 55–60), dibutyltin dichloride (hR_f 70–75) and tributyltin methoxide (hR_f 80–85) appeared in long-wavelength UV light ($\lambda = 365$ nm) as yellow fluorescent chromatogram zones on a dark background. The detection limits (calculated as Sn) lay between 5 and 10 (dimethyltin dichloride, dibutyltin dichloride, tributyltin methoxide) and 50 (trimethyltin chloride) substance per chromatogram zone.

In situ quantitation: Fluorimetric measurements were made by exciting at $\lambda_{exc} = 365$ nm and measuring the fluorescence emission at $\lambda_{em} > 560$ nm (cut off filter FI 56).

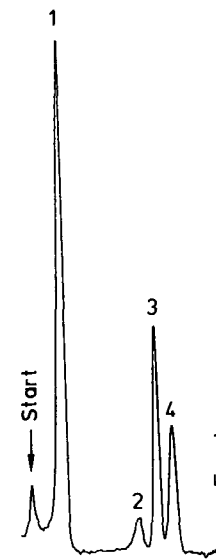


Fig. 1: Fluorescence scan of a chromatogram track with 100 ng substance (calculated as Sn) per chromatogram zone: 1 = dimethyltin dichloride, 2 = trimethyltin chloride, 3 = dibutyltin dichloride, 4 = tributyltin methoxide.

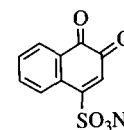
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1,2-Naphthoquinone-4-sulfonic Acid Reagent (Folin's Reagent)

Reagent for:

- Amino acids, peptides [1]
- Aromatic amines [1, 4, 5]
e.g. parsalimide [6]
- Piperidine derivatives [7]
e.g. taurinopiperidine
- Aliphatic amines [8]
- Ergot alkaloids, LSD [9]
- Phenolic aromatic sulfides, sulfoxides, sulfones [10]
- Diuretics [11]
e.g. chlorthalidon, clorexolone, cyclopentiazide



$C_{10}H_5NaO_5S$

$M_r = 260.20$

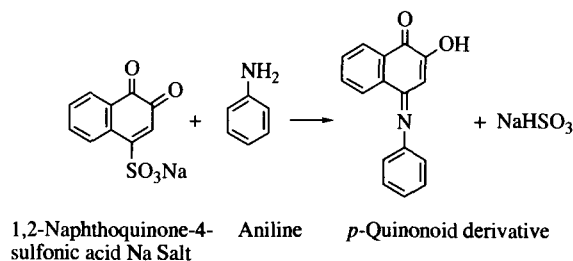
Preparation of the Reagent

Dipping solution For aromatic amines: Dissolve 0.5 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 30 ml water and add 65 ml ethanol and 5 ml acetic acid [5].

Spray solution	<p><i>For amino acids:</i> Dissolve 0.2–0.3 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 100 ml aqueous sodium carbonate solution (5–10%) [1].</p> <p><i>For aromatic amines:</i> Dissolve 0.5 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 95 ml water and treat with 5 ml glacial acetic acid [1, 6]; if necessary, filter off the insoluble part [1].</p> <p><i>For aliphatic amines:</i> Dissolve 0.6 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 12 ml water, make up to 200 ml with ethanol (90%) and add 10 ml pyridine [8].</p>
Storage	The dipping solution may be stored in the refrigerator for several days [5], the spray solution for amino acids should always be made up fresh [1].
Substances	1,2-Naphthoquinone-4-sulfonic acid sodium salt Ethanol Acetic acid (100%) Sodium carbonate decahydrate

Reaction

Primary amines and substances with reactive methylene groups react with 1,2-naphthoquinone-4-sulfonate to yield intensely colored *p*-quinoid derivatives, which, in the case of aryl amines, are indophenol dyes [12, 13].



Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution until the layer begins to be transparent and then dried in a stream of cold air [1].

After a few minutes variously colored chromatogram zones appear on a yellow background.

Note: It is possible to differentiate amino acids by color on the basis of the marked different shades produced [2, 3]. Proline and hydroxyproline, that only react weakly with ninhydrin, also yield pink-red colored derivatives [2]. Ergot alkaloids and LSD detected by spraying with 10% hydrochloric acid and then heating to 110 °C for 20 min after they have been treated with the reagent [9]. Ergot alkaloids and LSD yield red purple zones when treated in this manner; other alkaloids, e.g. reserpine, emetine, cocaine, strychnine, pilocarpine, atropine, scopolamine, cocaine and opium alkaloids, do not give a reaction [9].

In the case of diuretics the chromatogram is first sprayed with sodium hydroxide solution (*c* = 1 mol/L) and then with a saturated solution of 1,2-naphthoquinone-4-sulfonic acid sodium salt in ethanol – water (50+50) [11]. Stable orange-colored chromatogram zones appear over a period of ca. 15 min, their intensity increases on storage in the dark (1–2 days) [11].

Amino acids yield various colors [1].

Heating the chromatograms after treatment is not generally to be recommended, it leads to characteristic color changes in some cases [4].

The detection limits per chromatogram zone are 1–3 µg substance for ergot alkaloids [9], 5 µg for diuretics [11] and 5–30 ng for aromatic amines [5].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, aluminum oxide, polyamide and cellulose layers.

Procedure Tested

Aromatic Amines [5]

Method	Ascending, one-dimensional, stepwise development (5 min intermediate drying in a stream of cold air) in a trough chamber at 20°C without chamber saturation.
Layer	HPTLC plates Silica gel F ₂₅₄ (MERCK), which had been prewashed before use by complete immersion overnight in 2-propanol and then dried at 110°C for 30 min.
Mobile phase	1. Methanol – glacial acetic acid (100+1). 2. Chloroform – <i>n</i> -hexane – diisopropyl ether – dichloromethane – formic acid (50+35+10+5+0.45).
Migration distance	1. 0.8 cm 2. 6.5 cm
Running time	1. 1 min 2. 25 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed in the dipping solution for 2 s and then dried in stream of cold air.

After a few minutes 2,4-diamino-6-methylphenol (hR_f 5–10), 3-chloro-4-methoxyaniline (hR_f 25–30), aniline (hR_f 35–40), 4-bromoaniline (hR_f 40–45), 3-chloroaniline (hR_f 50–55), 2,6-dimethylaniline (hR_f 60–65), 2-methyl-6-ethylaniline (hR_f 65–70) and 2-chloroaniline (hR_f 70–75) yielded orange-colored chromatogram zones on a yellow background. The detection limits were between 5 ng (2,4-diamino-6-methylphenol) and 30 ng (2,6-dimethylaniline) substance per chromatogram zone.

In situ quantitation: The absorption photometric analysis in reflectance was carried out at the wavelength $\lambda = 510$ nm (Fig. 1).

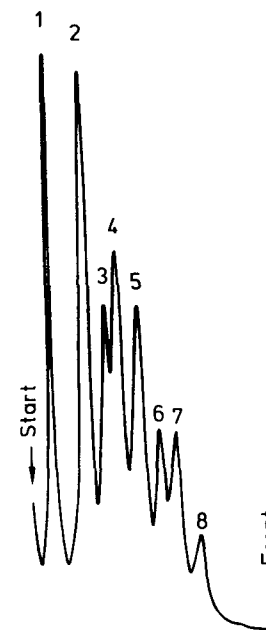


Fig. 1: Reflectance scans of a chromatogram track with 100 ng each of: 1 = 2,4-diamino-methylphenol, 2 = 3-chloro-4-methoxyaniline, 3 = aniline, 4 = 4-bromoaniline, 5 = 3-chloroaniline, 6 = 2,6-dimethylaniline, 7 = 2-methyl-6-ethylaniline and 8 = 2-chloroaniline.

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arylamines [9]
 imipramine, desipramine [10, 11]
 xanthene derivatives [12]
 diazepam [2]

- Testosterone [2]
- Acetylacetonates
 e.g. of Be, Al, V, Fe, Ce, Mn [2]
- Sugars
 e.g. fructose, glucose [2]
- 1,1'-Carbonyldiimidazole [2]
- Phospholipids
 e.g. lecithin, sphingomyelin [14]

HNO₃
 M_r = 63.01

Nitric Acid Vapor Reagent (Nitrous Fumes/Nitric Oxide Reagent)

Reagent for:

- Aromatics and compounds with aromatic substituents
 e.g. ephedrine [1, 2]
 methylephedrine, oxeladin citrate [1]
 catecholamine and serotonin metabolites [3]
 insecticides: rotenone, elliptone, deguelin, tephrosin [4]

Preparation of the Reagent

Reagent solution	Fuming nitric acid.
Storage	Fuming nitric acid may be stored for several months in a tightly sealed brown glass bottle.
Substances	Nitric acid, fuming (100%)

Reaction

Under the chosen conditions aromatic compounds are nitrated to nitroaromatics [1]. The detection of rotenone [1] (see below) depends on the reduction of silver ions, incorporated into the layer, to metallic silver in the presence of ammonia [4]. The mechanism of the reaction of many substances leading to fluorescent derivatives has not yet been elucidated [2].

Method

The chromatogram is freed from mobile phase in a stream of warm air and placed layer down for 40–45 s [6, 7], 1 min [5, 8], 3 min [1, 4], 10 min [1, 14] or 30–60 min [11] in a conditioning chamber containing 40 ml fuming nitric acid. The chromatogram is then freed from excess nitrous fumes for 5 min in a stream of cold air and occasionally heated to 160 °C [1, 2] or 180 °C [14] for 15 min. In the case of the rotenone insecticides (silver nitrate-impregnated layer!) the chromatogram is exposed to an atmosphere of ammonia for 10 min after it has been reacted with nitrous fumes [4], while for xanthene derivatives reaction is followed by irradiation with short-wavelength UV light ($\lambda = 254$ nm) for 5 min [12].

Aromatic compounds generally yield yellow to brown chromatogram zones that usually absorb UV light at $\lambda = 270$ nm [1]. These compounds can frequently be excited to fluoresce by long-wavelength UV light ($\lambda = 365$ nm) [2, 12]. Rotenoides yield dark [4], arylamines, e.g. imipramine and desipramine, pale yellow to brown-red [9, 11] and catecholamines yellow chromatogram zones on a pale background [3]. A whole range of substances, e.g. xanthene derivatives, diazepam, testosterone, glucose, fructose,

ephedrine etc., fluoresce yellow or blue when excited by long-wavelength UV light ($\lambda = 365$ nm) [2, 12].

Note: Nitrous fumes can also be generated in a twin trough chamber e.g. by pouring 50% nitric acid [12], hydrochloric acid ($c = 2$ mol/L) [11] or sulfuric acid onto sodium nitrite.

It can be advantageous to heat the chromatogram to 160 °C for 15 min before treating with nitrous fumes and to place it in the reagent chamber while still hot [1]. Heat to 260 °C has even been recommended for the purpose of reducing the fluorescent background [14], whereby the layer is previously immersed in 1 percent Ludox solution (silicic acid sol) to increase its stability [2]. The fluorescence of the substances detected usually remains stable for at least 2 weeks [2].

Brief exposure to nitrous fumes (up to 3 min) leaves the fluorescent power of acid-instable fluorescence indicator F_{254} , incorporated into most TLC layers, largely unaffected, so that the nitroaromatics so formed can be detected as dark zones on green fluorescent background [1]. For purposes of in situ quantitation it is recommended that the fluorescence indicator be destroyed by 10 min exposure to nitrous fumes in order to avoid difficulties in the subsequent evaluation [1].

The visual detection limits per chromatogram zone are 2.5 to 3.5 μg for pyrazolone compounds [6], 1–2.5 μg for arylazothiazoles and arylazopyrimidinylpyrazoles [7] and 2.5–4 μg for benzothiazoles [8]. Nanogram quantities can be detected by photometric methods [10, 14].

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers as well as on aluminium oxide, cellulose or chiral layers. Neither do difficulties occur on RP 18, Diol, NH_2 and CN phases. Silver nitrate- [4] and calcium oxalate-impregnated layers [9] are also suitable. However, polyamide phases are colored yellow.

Danger: Fuming nitric acid is very aggressive; eye protection and rubber gloves should always be worn!

Procedure Tested

Fungicides [13]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene – <i>n</i> -hexane – diisopropyl ether – ethyl acetate (67+20+8+5).
Migration distance	7 cm
Running time	15 min

Detection and result: The dried chromatogram (5 min in a stream of cold air) was heated to 160°C for 15 min and placed, while still hot, for 10 min in the empty half of a twin trough chamber whose other trough contained 10 to 15 ml fuming nitric acid. After exposure the chromatogram was heated to 160°C for 15 min to remove excess nitric oxide. Scanning was carried out after cooling. It was not possible to make a visual check of the fungicides ($\approx 1 \mu\text{g}$ each) iprodione (hR_f 10–15), procymidon (hR_f 35–40) and vinclozolin (hR_f 55–60) investigated, due to the yellow coloration of the chromatogram zones.

In situ quantitation: The absorption photometric scan in reflectance was carried out at $\lambda = 270 \text{ nm}$ (Fig. 1).

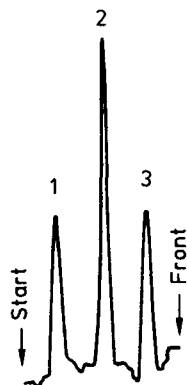


Fig. 1: Reflectance scan of a chromatogram track with $1 \mu\text{g}$ each per chromatogram zone: 1 = iprodione, 2 = procymidon, 3 = vinclozolin.

References

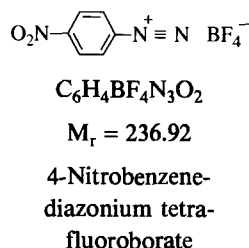
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4-Nitrobenzenediazonium Tetrafluoroborate Reagent

Reagent for:

- Phenols [1–7]
 - e.g. plant phenols [8]
 - estrogens [9]
 - procyanidins [10]
 - catecholamines [11]
- Zearalenone [12]
- Aromatic amines [13]
 - e.g. diphenylamine (anti-ageing additive) [14]
- Pesticides
 - e.g. carbamate insecticides [1, 4, 7, 8, 15–19]
 - such as desmedipham, dioxacarb, phenmedipham, propoxur, pyrazon, aminocarb, aldicarb, carbofuran, carbaryl, oxamyl
 - e.g. carbamate herbicides [21]
 - such as propanil
 - e.g. organophosphorus insecticides [4, 20]
 - such as parathion, parathion methyl, coumaphos, dichlorvos, fenitrothion

NaOH
M_r = 40.00
Sodium hydroxide



Preparation of the Reagent

Dipping solution I Dissolve 2 g sodium hydroxide [20] or 2.8 g potassium hydroxide [15] in 5 ml water and make up to 50 ml with ethanol or methanol.

Dipping solution II Dissolve 25–50 mg 4-nitrobenzenediazonium tetrafluoroborate in 10 ml diethylene glycol and make up to 100 ml with water [20] or acetone [15].

Spray solution I Dissolve 4–15 g potassium hydroxide [1, 4, 7, 16–18] or sodium hydroxide [8] in 100 ml methanol, ethanol or water.

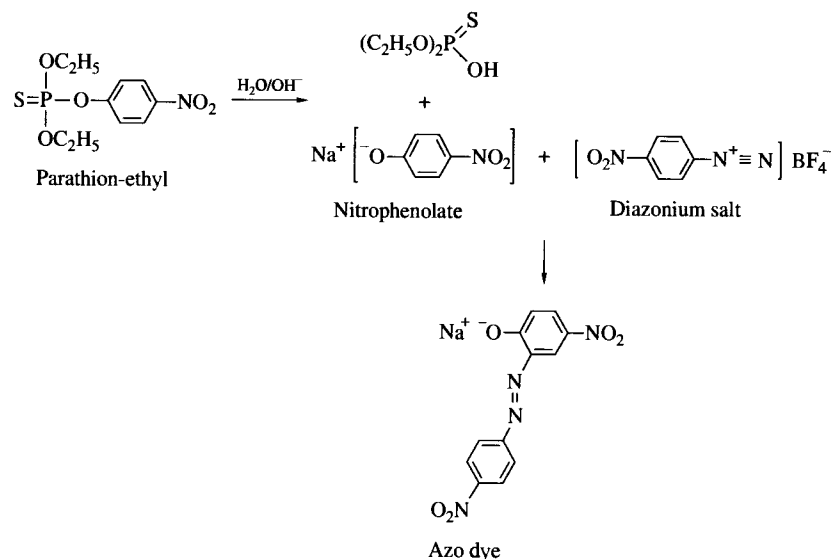
Spray solution II Dissolve 10 to 100 mg 4-nitrobenzenediazonium tetrafluoroborate in 100 ml methanol [7, 17, 18]. Alternatively it is possible to use 0.5 to 2% solutions in acetone [16], acetone – methanol (50+50) [1] or 50% acetic acid [9]. A saturated solution in ethanol – diethylene glycol (90+10) can also be used [8].

Storage The dipping and spray solutions I can be stored over longer periods. The dipping and spray solutions II may only be stored for a few hours with cooling and should, therefore, always be made up freshly before use [7].

Substances Potassium hydroxide, pellets
Sodium hydroxide, pellets
4-Nitrobenzenediazonium tetrafluoroborate
Diethylene glycol
Ethanol
Methanol
Acetone

Reaction

The hydrolytic action of alkalis on the thiophosphate insecticides, such as parathion, yields free phenols that then couple with the diazonium salt to yield azo dyes.



Method

The chromatograms are freed from mobile phase in a stream of warm air for 3 min, then immersed in dipping solution I for 3–5 s or uniformly sprayed with spray solution I. Then, except in the case of thiophosphate insecticides, the plate is immediately immersed in dipping solution II for 3–5 s or homogeneously sprayed with spray solution II. In the case of thiophosphate insecticides there is a delay of 2 min [4] or the plates are heated to 70–110 °C for 10–15 minutes before the plates are exposed to the second reagent solution.

This yields variously colored chromatogram zones on a colorless background. The zones of phenols are reddish to blue-violet [4, 7, 8].

Note: The first reagent treatment with alkali can be omitted in the case of phenols (e. g. estrogens) [2, 3, 5, 8, 9]. Dipping is preferable to spraying since it yields darker chromatogram zones and, hence, improves the detection sensitivity [15]. The presence of diethylene glycol in the reagent has a favorable effect on the color intensity and stability of the derivatives [15]. Most thiophosphate insecticides do not give any reaction [4].

The detection limits for catecholamines are 10 to 50 ng substance per chromatogram zone [11] and 50 ng substance per chromatogram zone for carbaryl and a series of other

carbamate insecticides [8, 15]. The detection sensitivity is generally better on silica g than on aluminium oxide layers [4]. It is possible to detect parathion with a sensitivity of 5 to 50 pg after it has been oxidized with bromine to paraoxon, by combination with an enzyme inhibition reaction (choline esterase, substrate: 1-naphthyl acetate). White zones of inhibition are produced on a blue-red background [2, 3].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr or Si 50000 layers.

Procedure Tested

Thiophosphate Insecticides [20]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ , extra thin layer (MERCK); before the samples were applied these were immersed overnight in 2-propanol and then dried for 30 min at 110 °C.
Mobile phase	<i>n</i> -Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (908 + 40 + 26 + 25 + 1).
Migration distance	8 cm
Running time	30 min

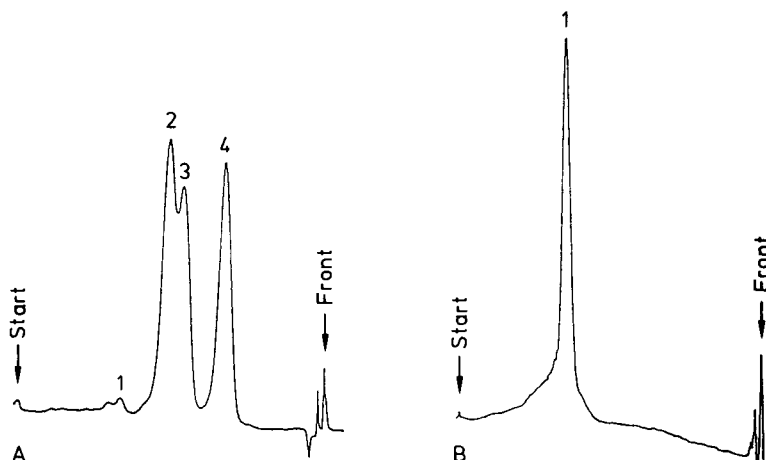
Detection and result: The chromatogram was freed from mobile phase in a stream of warm air for 3 min, immersed in dipping solution I for 3 s, heated to 110 °C for 15 min and then immersed in dipping solution II for 3 s.

After drying in a stream of cold air coumaphos (*hR_f* 30–35) appeared as an intense red chromatogram zone on a colorless background, while parathion methyl (*hR_f* 40–45), fenitrothion (*hR_f* 45–50) and parathion ethyl (*hR_f* 60–65) yielded yellow zones as they did with sodium hydroxide alone (*q.v.*). The detection limit for coumaphos was 10 ng per chromatogram zone.

In situ quantitation: The absorption photometric scan in reflectance of parathion methyl, fenitrothion and parathion ethyl was carried out at a mean wavelength $\lambda_{\text{max}} = 406 \text{ nm}$ (Fig. 1A). Coumaphos was determined at $\lambda = 540 \text{ nm}$ (Fig. 1B).

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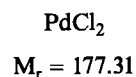


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Palladium(II) Chloride Reagent

Reagent for:

- Thiophosphate esters [1–4]
e.g. insecticides [5–23]
such as dimethoate, bromophos-ethyl, Fac, Rogor, parathion, chlorthion, Meta-Systox, diazinon, propetamphos, malathion, terbufos, azinphos-methyl, demeton, phorate, disulfoton, vamidothion
- Sulfur compounds
e.g. mercaptans, sulfides (thioethers), disulfides [24] polysulfides [25] phenothiazines [4]
- Antioxidants
e.g. esters of gallic acid, dialkyl- and diaryldithiophosphates [26, 27]



Preparation of the Reagent

- Dipping solution** Dissolve 500 mg palladium(II) chloride in 2.5 ml hydrochloric acid (32%) and make up to 100 ml with ethanol [10].
- Spray solution** Dissolve 250 mg to 5 g palladium(II) chloride in 100 ml ethanol [9], water [1], hydrochloric acid ($c = 0.2 \dots 1 \text{ mol/L}$) [3, 6, 8, 13, 15, 19–21, 23, 27], ethanolic hydrochloric acid [5] or hydrochloric acid – acetone (50+50) [25, 26].

- Storage** The dipping solution can be stored for ca. 1 month.
- Substances** Palladium(II) chloride
Hydrochloric acid (32%)
Ethanol
Acetone

Reaction

Palladium(II) chloride forms colored complexes with many aromatic and sulfur-containing compounds [27].

Method

The chromatograms are freed from mobile phase for 3 min in a stream of warm air, immersed for 2 s in the dipping solution [10] or homogeneously sprayed with the spray solution [9], dried in a stream of warm air and then heated to 110–120 °C for 10–20 min [9, 10].

Colored zones are formed sometimes without heating [1, 3, 6, 7, 13–15, 18, 20] organophosphorus insecticides forming yellow-brown [2, 6, 9, 10, 14, 18, 28] to black chromatogram zones on a colorless [14, 23] to pale yellow [2] or light brown [6, 10] pinkish grey [9] background. Mercaptans, sulfides, disulfides and polysulfides appear white, yellow, orange and brown [24, 25] and antioxidants yellow, grey, brown, pink or violet [27].

Note: It is occasionally recommended that sodium acetate be added to the reagent [1]. Thiophosphate insecticides with a simple P–S bond yield yellow chromatogram zones and those with a P=S double bond yield brown ones on a light brown background [1]. Further treatment of the stained chromatogram with iodine vapors increases the detection sensitivity [7] more than does spraying afterwards with caustic soda solution, which is also occasionally recommended [16, 17, 20, 21].

The detection limits for compounds with P=S double bonds are lower than those for substances with single P–S bonds [7]. They are lower on silica gel than on polyamide layers [15] and are, for instance, 10–20 ng substance per chromatogram zone [15].

organophosphorus pesticides [10]. Higher levels of up to 5 µg are regularly reported in the literature [6, 7, 15, 17].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000, polyamide and RP layers.

Procedure Tested

Organophosphorus Insecticides [10, 28, 29]

Method	Ascending, one-dimensional, two-fold development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	1. Chloroform — diethyl ether — <i>n</i> -hexane — toluene (29.3 + 25.7 + 25 + 20). 2. Ethyl acetate
Migration distance	1. 6 cm 2. 1 cm
Running time	1. 20 min 2. 5 min

Detection and result: The chromatogram was dried for ca. 3 min in a stream of warm air, immersed in the dipping solution for 2 s, dried in a stream of warm air for 3 min and then heated to 110 °C for 10 min in the drying cupboard.

Demeton-S-methyl sulfone (hR_f 0–5), dimethoate (hR_f 5–10), demeton-S-methyl (hR_f 20–25), triazophos (hR_f 40–45), azinphos-methyl (hR_f 40–45), azinphos-ethyl (hR_f 50–55), malathion (hR_f 60–65), parathion-methyl (hR_f 75–80) and parathion-ethyl (hR_f 80–85) yielded yellow to brown chromatogram zones on a light brown background, with thiophosphate insecticides with P=S double bonds appearing as brown zones and those with single P–S bonds as yellow zones.

In situ quantitation: The absorption photometric quantitation was carried out in reflectance at a mean wavelength of $\lambda = 370$ nm. The detection limits per chromatogram zone lay between 10 ng for dimethoate and 20 ng for parathion-ethyl.

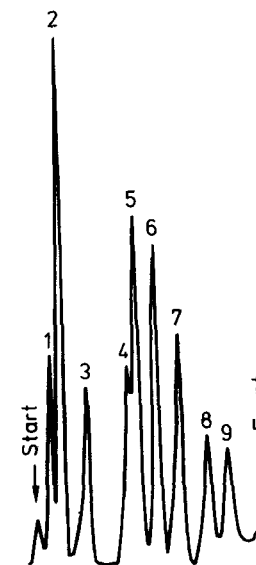


Fig. 1: Reflectance scan of a chromatogram track with 100 ng each substance per chromatogram zone: 1 = demeton-S-methyl sulfone, 2 = dimethoate, 3 = demeton-S-methyl, 4 = triazophos, 5 = azinphos-methyl, 6 = azinphos-ethyl, 7 = malathion, 8 = parathion-methyl and 9 = parathion-ethyl.

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Phosphoric Acid Reagent

Reagent for:

- Sterols, steroids [1–7]
 - e.g. cholesterol [8]
 - aldosterone, hydrocortisone, androsterone, estradiol [8]
 - contraceptives [9, 10]
 - 17-spirosteroids [11]
 - trenbolone [12]
 - liquid crystals [13]
- *Digitalis* glycosides [14, 15]
- Indole derivatives
 - e.g. tryptophan, indole-3-acetic acid [16]
 - Amanita* toxins [17]
- Quinoxalone derivatives of α -ketoacids
 - e.g. pyruvic acid [18]
- Components of edible oils (lipids)
 - e.g. in groundnut oil, castor oil [19]



$$M_r = 98.00$$

Preparation of the Reagent

- | | |
|-------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Dipping solution | Mix 50 ml <i>ortho</i> -phosphoric acid (85%) carefully with 50 methanol under cooling. |
| Spray solution | A solution of 10 to 50% <i>ortho</i> -phosphoric acid in water [1–6, 11, 13, 17, 18], methanol [9, 17, 19], ethanol [12, 16, 17, 20], a tone [17] or carbon tetrachloride – <i>n</i> -propanol (3+2) [14]. |

Storage	Both reagent solutions may be stored, cool and in the dark, several days.
Substances	<i>ortho</i> -Phosphoric acid (85%) Methanol Ethanol

Reaction

The mechanism of the reaction has not been elucidated [16].

Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the reagent solution for 1 to 2 s or homogeneously sprayed with it until the layer begins to be transparent, then after drying in a stream of warm air it is heated to 105–120 °C for 5–30 min and occasionally (tryptophan derivatives) [16] for 40 min. In exceptional cases evaluation is made without heating (trenbolone [12]).

Various colored chromatogram zones (grey, blue, brown, orange, violet) are produced on a pale background; the zones frequently fluoresce intensely on exposure to long-wavelength UV light ($\lambda = 365$ nm).

For instance, trenbolone fluoresces yellow [12] and the qinoxalone derivative of pyruvic acid yellow-green [18]. In the case of steroids and *Digitalis* glycosides it is possible to differentiate on the basis of various fluorescence colors [8, 9, 15].

Note: Like sulfuric acid (*q.v.*) *ortho*-phosphoric acid is a universal reagent, with which almost all classes of substance can be detected at high temperatures (150–180 °C) by charring: e.g. high molecular weight hydrocarbons (mineral oils) [20]. The colors and fluorescences produced at lower temperatures (<120 °C) and their intensities are very dependent on the temperature and period of heating. It is not possible to use meta- or pyrophosphoric acid in place of *ortho*-phosphoric acid, since, for instance, *amanita* toxins react well with alcoholic phosphoric acid only weakly with aqueous phosphoric acid and not at all with meta- or pyrophosphoric acid [17].

The fluorescence can be stabilized by dipping the chromatograms in liquid paraffin – *n*-hexane (1+2) [21] or paraffin – carbon tetrachloride (1+9) [14]. Quantitative evaluation must generally be carried out rapidly since neither the colors nor the fluorescences are fast [16, 17]. Colored chromatogram zones can be preserved over a long period by covering the chromatogram with a glass plate [9].

Detection with phosphoric acid at room temperature (with no heating afterwards) is specific for trenbolone, since related steroids such as progesterone and testosterone do not interfere under these conditions [12].

The detection limits per chromatogram zone are below 1 µg for steroids [9] (e.g. 250 pg for trenbolone [12]) and 500 ng for indole derivatives [16].

The reagent can be used on silica gel, silver nitrate-impregnated silica gel [19] kieselguhr, Si 50000, RP, Diol and NH₂ layers. Cellulose layers are less suitable because after application of the reagent the background itself fluoresces strongly, so that fluorescence emission can only be detected after the application of large quantities of substance per chromatogram zone.

Procedure Tested

Steroids [21, 22]

Method	Ascending, one-dimensional development in a trough chamber without (A) or with chamber saturation (B).
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that were prewashed before application of the sample, by developing once to the upper edge of the plate with chloroform – methanol (50+50), and then dried at 110 °C for 30 min. In the case of example A. the layer was conditioned to 0% rel. humidity in a conditioning chamber (over conc. sulfuric acid) after sample application.
Mobile phase	A. Cyclohexane – diethyl ether (50+50). B. Chloroform – methanol (98+2).
Migration distance	A. 6 cm B. 8 cm
Running time	15 min

Detection and result: The chromatogram was first dried in a stream of cold air, immersed in the reagent solution for 1 to 2 s and then heated to 120–125 °C for 15–20 min. Blue-grey to violet chromatogram zones were produced on a colorless background that could be excited to fluoresce various colors with long-wavelength UV light ($\lambda = 365$ nm).

On a dark background cholesterol (Eluent A, hR_f 20–25) emitted blue, coprostanol (Eluent A, hR_f 25–30) blue, 4-cholesten-3-one (Eluent A, hR_f 40–45) blue, 5 α -cholestan-3-one (Eluent A, hR_f 60) blue, coprostanone (Eluent A, hR_f 70) blue, estriol 3-sulfate (Eluent B, hR_f 5–10) yellow, 11-ketoetiocholanolone (Eluent B, hR_f 15–20) blue, estrone (Eluent B, hR_f 20–25) ochre, 11-desoxycorticosterone (Eluent B, hR_f 30–35) yellow, 17 α -ethinyl-5-androstene-3 β ,17 β -diol (Eluent B, hR_f 45–50) ochre, 4-cholesten-3-one (Eluent B, hR_f 55–60) faint blue and coprostanone (Eluent B, hR_f 65–70) violet fluorescences.

The detection limits lay between 5 ng and 50 ng substance per chromatogram zone.

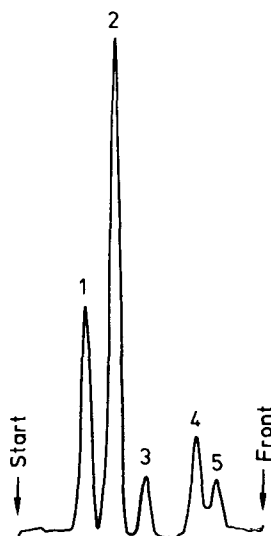


Fig. 1

Fig. 1: Fluorescence scan of a chromatogram track with 255 ng cholesterol (1), 535 ng coprostanol (2), 310 ng 4-cholesten-3-one (3), 320 ng 5 α -cholestan-3-one (4) and 220 ng coprostanone (5) per chromatogram zone.

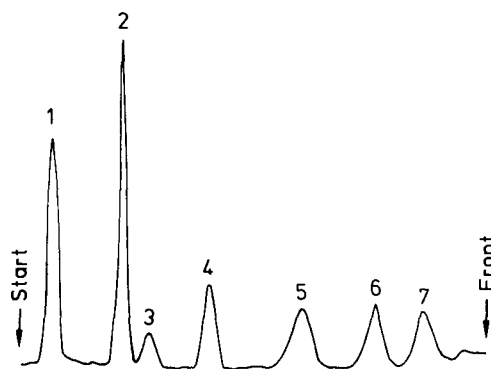


Fig. 2

Fig. 2: Fluorescence scan of a chromatogram track with 100 ng each of estriol-3-sulfate (1), 11-ketoetiocholanone (2), estrone (3) 11-desoxycorticosterone (4) and 17 α -ethinyl-5-androsten-3 β ,17 β -diol (5), together with 1 μ g each of 4-cholesten-3-one (6) and coprostanone (7) per chromatogram zone.

The reagent is not suitable for quantitative determinations because the fluorescences are not stable: In Fig. 1 liquid paraffin – *n*-hexane (1 + 2) was used to stabilize the fluorescence.

In situ quantitation: The fluorimetric evaluation was carried out with excitation $\lambda_{exc} = 365$ nm and fluorescence emission was measured at $\lambda_{fl} > 430$ nm (cut off FI 43).

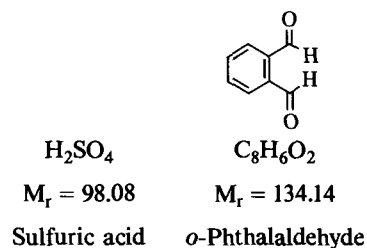
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o-Phthalaldehyde– Sulfuric Acid Reagent

Reagent for:

- Ergot alkaloids and derivatives [1, 2]
e.g. lysergic acid, LSD, ergotamine, ergocristine
e.g. hydrogenated ergot alkaloids
such as dihydrolysergic acid, dihydroergotamine
dihydroergocristine, dihydroergotoxin
- β -Blockers [3]
e.g. propranolol, alprenolol, nadolol
- Histidylpeptides [3]
- Indole derivatives
e.g. tryptophan, tryptamine,
serotonin, bufotenin



Preparation of the Reagent

Dipping solution Dissolve 0.5–1 g *o*-phthalaldehyde (phthaldialdehyde, OPA) in 45 ml methanol and cautiously add 5 ml sulfuric acid (95–97%) [3].

Spray solution Dissolve 0.2 g *o*-phthalaldehyde cautiously in 100 ml sulfuric acid (95–97%) [1].

Storage The dipping solution is stable for several weeks at room temperature [3], the spray solution may be stored in the refrigerator 2–3 days [1].

Substances Phthaldialdehyde
Sulfuric acid (95–97%)
Methanol

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immerse in the dipping solution for 3 s or sprayed homogeneously with the spray solution: – in the case of the β -blockers – heated to 80 °C for 3 min.

β -Blockers yield yellow to pink-colored chromatogram zones on a colorless background, which, like the zones of the ergot alkaloids and hydrogenated ergot alkaloids, can usually be excited to blue fluorescence on irradiation with long-wavelength UV light ($\lambda = 365 \text{ nm}$) [1, 3].

Note: *o*-Phthalaldehyde in the presence of mercaptoethanol or cysteine has already been discussed as a reagent [4]. The present monograph describes the use of *o*-phthalaldehyde in the presence of sulfuric acid. There are, in addition, a number of applications, which have been described, employing *o*-phthalaldehyde without any additions, e.g. for the detection of primary arylamines, histamine, histidine and histidylpeptides [5–7].

The natural fluorescence of ergot alkaloids is considerably augmented by the reagent [1]. Heating for longer than 3 min or to more than 80 °C leads to a reduction in fluorescence intensity and, hence, should be avoided [3].

The fluorescence intensity can be stabilized and enhanced by dipping chromatograms in a solution of liquid paraffin – *n*-hexane (1+2) [3]. The detection

limits for β -blockers, ergot and dihydroergot alkaloids and other indole derivatives in the lower nanogram range [1, 3, 8].

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers

Procedure Tested

β -Blockers [3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK), that were prewashed before application of the samples by developing to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110°C for 30 min.
Mobile phase	Methanol – ammonia solution (25 %) (99+1).
Migration distance	8 cm
Running time	20 min

Detection and result: The chromatogram was dried for 15 min in a stream of warm air, immersed in the dipping solution for 3 s and then dried at 80°C for 3 min in the drying cupboard. After cooling to room temperature it was immersed for 1 s in a solution of liquid paraffin – *n*-hexane (1+2) to enhance (by a factor of ca. 2) and stabilize the fluorescence intensity and then dried for 3 min in a stream of cold air.

4-Hydroxypropranolol (hR_f 40–45) and propranolol (hR_f 55–60) yielded yellow chromatogram zones on a colorless background that could be excited in long-wavelength UV light ($\lambda = 365$ nm) to orange fluorescence on a faint blue fluorescent background. The detection limits were 5 ng substance per chromatogram zone.

In situ quantitation: Fluorimetric measurements were made by exciting at $\lambda_{exc} = 436$ nm and measuring the fluorescence emission at $\lambda_{fl} > 560$ nm (cut off filter FI 56).

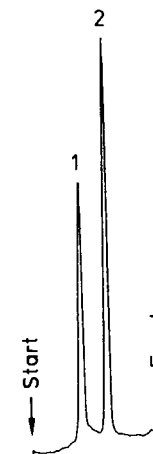


Fig. 1: Fluorescence scan of a chromatogram track with 50 ng each of 4-hydroxypropranolol (1) and propranolol (2) per chromatogram zone.

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Potassium Dichromate-Perchloric Acid-Nitric Acid-Sulfuric Acid Reagent (Forrest Reagent)

Reagent for:

- Antidepressives
e.g. imipramine derivatives
such as imipramine, clomipramine, desipramine, trimipramine [1]
- Neuroleptics
e.g. phenothiazine derivatives
such as chlorphenethazine, perazine, promazine [1]
- Antihistamines
e.g. phenothiazine derivatives
such as alimemazine [1]

$K_2Cr_2O_7$	$HClO_4$	HNO_3	H_2SO_4
$M_r = 294.19$	$M_r = 100.46$	$M_r = 63.01$	$M_r = 98.08$
Potassium dichromate	Perchloric acid	Nitric acid	Sulfuric acid

Preparation of the Reagent

Solution I	Dissolve 0.2 g potassium dichromate in 100 ml water.
Solution II	Perchloric acid (20%).
Solution III	Nitric acid (50%).
Solution IV	Sulfuric acid (30%).

Spray solution	Carefully mix equal volumes of solutions I to IV.
Dipping solution	Dilute 10 ml of the spray solution with 90 ml water.
Storage	The reagent solutions may be stored over long periods.
Substances	Potassium dichromate Perchloric acid (60%) Nitric acid (65%) Sulfuric acid (95-97%)

Reaction

The mechanism of the reaction is not known. Detection probably depends on the reversible formation of colored radicals [2, 3].

Method

The chromatograms are dried in a stream of cold air, immersed in the dipping solution for 1 s or sprayed homogeneously with the spray solution until the layer becomes transparent and then dried in a stream of cold air for 5 min.

Imipramine and its derivatives produce blue, phenothiazines blue, violet, red orange to skin-colored chromatogram zones, that fade relatively quickly, on a colorless background (Fig. 1).

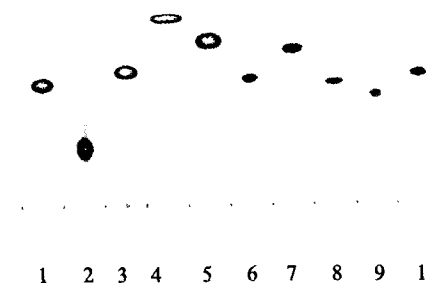


Fig 1: Chromatogram of imipramine and phenothiazine derivatives after staining with FORREST reagent [4]: 1 = imipramine, 2 = desipramine, 3 = clomipramine, 4 = lofepramine, 5 = trimipramine, 6 = thioridazine, 7 = chlorphenethazine, 8 = periciazine, 9 = promazine, 10 = promethazine

Table 11 Colors of chromatogram zones after treatment with FORREST reagent [1].

Color	Substance			
Edge blue, center pale	clomipramine desipramine	imipramine	lofepramine	trimipramine
Red, orange, pink	alimemazine antazoline bromhexine chlorphenethazine clozapine	dibenzepine dixyrazine fluphenazine fluspirilene homofenazine	oxypertine perazine periciazine perphenazine promazine	promethazine prothipendyl trifluoperazine triflupromazine viloxazine
Blue, violet	benperidole dihydroergotamine	imiclopazine levomepromazine	thiethylperazine	thioridazine
No coloration	acebutolol amfetaminile aminophenazone amitriptyline apomorphine articaine atropine atenolol bamipine benproperine benzatropine biperidene bisacodyl bromocriptine brompheniramine buphenine bupivacaine bupranolol	butalamine butamirate butanilicaine chlorazanine chloroquine chlorphenoxamine chlorprothixene chlorotheophylline cimetidine clemastine clenbuterol clobutinol clomethiazole clonidine clopamide codeine caffeine cyproheptadine	cytarabine dextromethorphan dextropropoxyphene diltiazem dimetindene dioxopromethazine diphenhydramine diphenylpyraline disopyramide dosulepine doxapram doxepine doxylamine drofenine eprazinone ethenzamide etozoline fendiline	fenetyline fenproporex fenyramidol flecainide flupentixol glymidine haloperidol lidoflazine melperone nortriptyline oxomemazine pheniramine pimozide pipamperone tiotixene trazodone trifluoperidol

Note The colors obtained are characteristic for the various substance classes (Table 1). Thus color tones obtained for phenothiazine derivatives are mainly reddish [1]. If the chromatogram zone contains more than 10 µg substance per spot there is only a colored outer ring surrounding an uncolored center (Fig. 1) [1, 4]. The FORREST reagent does not interfere with subsequent detection with the DRAGENDORFF reagent [1, 4].

The detection limits are in the range 100 to 600 ng substance per chromatogram zone [4, 5].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, RP, CN, NH₂, Diol, polyamide and cellulose layers.

Procedure Tested

Dibenzoazepine and Phenothiazine Derivatives [4, 5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Ethyl acetate – methanol – ammonia solution (25 (85+10+5).
Migration distance	7 cm
Running time	10–15 min

Detection and result: The chromatogram was dried for 15 min in a stream of cold air, immersed in the dipping solution for 1 s and then dried in a stream of cold air 5 min.

Immediately after dipping perphenazine (hR_f 28–33) appeared as pink-colored, fluphenazine (hR_f 30–35), dixyrazine (hR_f 33–38) and verophene (= promazine, hR_f 38–43) as skin-colored and periciazine (hR_f 35–40) as orange, promethazine (hR_f 43–48) as bright pink, alimemazine (hR_f 60–65) as skin-colored and dibutyl (ethyl) propazine, hR_f 75–80) as pink-colored chromatogram zones on a colorless background (Fig. 2). The colors faded after some time.

The detection limits of imipramine and its derivatives were 100 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 530$ nm (Fig. 3).

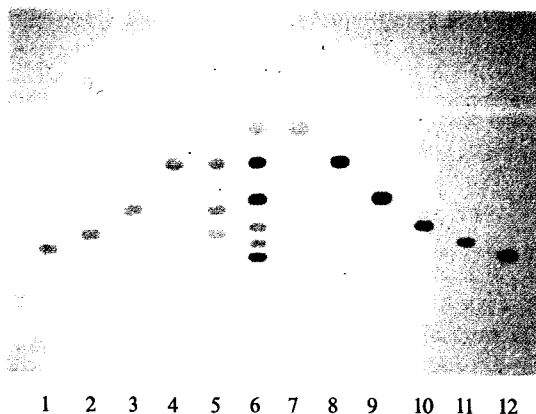


Fig 2: Chromatogram of dibenzoazepine and phenothiazine derivatives after staining with FORREST reagent: 1 = fluphenazine, 2 = periciazine, 3 = promethazine, 4 = alimemazine, 5 = mixture of substances 1 to 4, 6 = mixture of substances 7 to 12, 7 = dibutil, 8 = levomepromazine, 9 = chlorpromazine, 10 = verophene, 11 = dixyrazine, 12 = perphenazine.

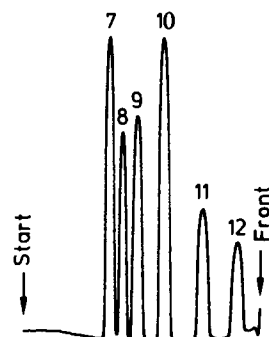


Fig. 3: Reflectance scan of chromatogram track 6 of Figure 2 with 4 μg substance per chromatogram zone each of 7 = dibutil, 8 = levomepromazine, 9 = chlorpromazine, 10 = verophene, 11 = dixyrazine, 12 = perphenazine.

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Potassium Hexaiodoplatinate Reagent

Reagent for:

- Organic nitrogen compounds [1–3]
 - e.g. drug substances and metabolites [4–9]
 - such as benzodiazepines [10, 11], polamidon, dolantin, dilaudid [12]
 - carbamazepine, maprotiline, methadone, methaqualone [13]
 - phencyclidine [14], imipramine, desimipramine [15]
 - morphine [16], naloxone [17], naltrexone [18]
 - codeine, 6-acetylmorphine [19], oxaflozane [20]
 - pentazocine, tripeleennamine [21], chloroquine [22]
 - e.g. alkaloids [23–25]
 - in *Antirrhinum* [26, 27], *Corydalis lutea* [28]
 - Hydastis canadensis* [29], *Thalictrum polygamum* [30]
 - Cinchona ledgeriana* [31]
 - such as ajmaline, atropine, ergotamine, raubasine, tropine [23]
 - isocorydine, stylopine, bicuculline [28], thebaine [32]
 - hydrastine, berberine [29], strychnine [33], cocaine [34]
 - methylpalaudinium chloride [30]
 - e.g. quaternary ammonium compounds (surfactants)
 - such as bencetonium chloride [35]
 - e.g. urethanes
 - from the reaction of isocyanates with 1-(2-pyridyl)piperazine [36]
 - e.g. heroin [19, 34]
- Thiols, thioethers and sulfoxides [37–39]
 - e.g. sulfur-containing amino acids
 - such as cysteine, cystine, methionine [37]
 - e.g. antibiotics and derivatives
 - such as penicillin benzathine and -embonate salts [38]
 - amoxicillin, penicilloic and penicillic acids, ampicillin,
 - tritylpenicillin sulfoxide, benzylpenicillin sulfoxide [39]
- Ketosteroids [3]

Reagent for:

- Vitamins
 - e.g. vitamin D₃ (cholecalciferol), vitamin K₁ [40]
 - vitamin B₁ (thiamine) [41]
- Indandione derivatives
 - e.g. pindone, valone [40]
- Thiophosphate pesticides [42]

KI	H ₂ (PtCl ₆) · 6H ₂ O
M _r = 166.01	M _r = 517.92
Potassium iodide	Hexachloroplatinic acid hexahydrate

Preparation of the Reagent

Dipping solution	Mix 3 ml 10% hexachloroplatinic(IV) acid solution with 97 10% methanol and 100 ml 6% aqueous potassium iodide solution [43]. <i>Variant:</i> Mix 45 ml 10% aqueous potassium iodide solution with 2.5 ml 10% hexachloroplatinic(IV) acid solution and make up 200 ml with water (pH 2.52) [44].
Spray solution	Add 3 ml 10% hexachloroplatinic(IV) acid solution to 100 6% aqueous potassium iodide solution and dilute with 97 water [3, 8, 35, 36, 45].
Storage	The reagent solutions should be stored in brown bottles. They may be kept in the refrigerator for ca. 1 week [8].
Substances	Potassium iodide Hexachloroplatinic(IV) acid solution (10%) Methanol

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are dried in a stream of warm air, immersed in the dipping solution for 1 to 4 s or sprayed homogeneously with the spray solution and, if necessary, dried in a stream of warm air.

Chromatogram zones of various colors are produced [35], usually immediately, on a pale pink background [36, 39, 44]; they can sometimes also be detected under short-wavelength UV light [$\lambda = 254$ nm] [40]. The red coloration of the plate background can be very largely removed by washing out excess reagent with water or with 1% acetic acid [3, 44]. In some cases the colors of the chromatogram zones become deeper if they are heated to 115 °C for 5 min [40].

Alkaloids produce pale yellow, pink, green, brown, blue or violet zones [23]. Urethanes blue-green to dark violet zones [36]. Thiols and penicillin derivatives appear immediately as white zones and sulfoxides only after a few minutes as yellow to yellowish-blue zones on a reddish background [37, 39], which becomes deep purple on spraying with water [37].

Note: Tertiary amines and quaternary ammonium compounds yield stronger colors than primary amines [25]. The dipping solution can also be used as spray solution [44]. Other reagent compositions have also been reported in the literature [1, 3, 6, 12, 13, 15, 18, 21, 23, 41]. In some cases the reagents have been made up in acetone [38, 39], methanol [14] or ethanol [37] and/or acidified with hydrochloric acid [3, 33, 37–40]. The concentrations of hexachloroplatinic(IV) acid have been in the range of 0.05–0.4%, those of potassium iodide between 0.5 and 24% [46]. A spray solution containing 2% potassium iodide and 0.23% hexachloroplatinic(IV) acid hexahydrate in N-hydrochloric acid is reported to yield the best coloration results with respect to detection sensitivity and color differentiation in the detection of morphine, codeine, quinine, methadone and cocaine [46]. Acidic reagent solutions have been recommended for benzodiazepines [10, 11]. Sulfones do not react [39].

The detection limits in substance per chromatogram zone are 10 ng for urethanes [36], 10 ng – 1 µg for alkaloids and 50 ng – 1 µg for penicillin derivatives [39].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, RP 18. CN, Diol, polyamide and cellulose layers; NH_2 phases are not suitable – because they decolorize the reagent [44].

Procedure Tested 1

Brucine, Strychnine in Plant Extracts [43]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK), that had been prewashed before application of the samples by developing to the upper edge of the plate with chloroform – methanol [50+50] and then drying at 110 °C for 10 min.
Mobile phase	Acetone – toluene – ammonia solution (25%) (40+15+5).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried for 45 min in a stream of warm air (removal of ammonia!), immersed for 4 s in the dipping solution and dried in a stream of cold air.

Brucine (hR_f 30–35) appeared as a blue chromatogram zone and strychnine (hR_f 50–55) as a brown one on a reddish-brown background. The detection limits were lower than 5 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength of the absorption maximum for brucine $\lambda = 700$ nm (Fig. 1).

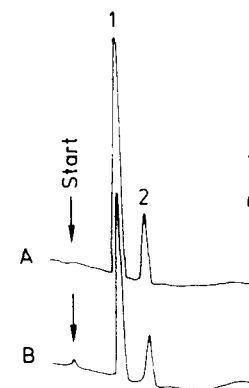


Fig 1: Reflectance scan of a chromatogram of *Nux vomica* extract (A) and of a reference trace (B) with 100 ng each of brucine (1) and strychnine (2) per chromatogram zone.

Procedure Tested 2

Opium Alkaloids [44]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 WF ₂₅₄ (MERCK).
Mobile phase	Acetone – toluene – ethanol – ammonia solution (25%) (40+40+6+2).
Migration distance	7 cm
Running time	10 min

Detection and result: The chromatogram was dried for 30 min at 100 °C and first immersed for 1 s in the dipping solution „variant“. Morphine (hR_f 10–15) and codeine (hR_f 15–20) yielded blue-violet chromatogram zones, narceine (hR_f 0), thebaine (hR_f 35–40), papaverine (hR_f 50–55) and narcotine (hR_f 65–70) yielded brown-violet chromatogram zones on a pale red background (Fig. 2A). There was a striking formation of pale half moon-shaped bands over the chromatogram zones in the direction of dipping when the chromatograms were dipped – but not when they were sprayed.

It is advisable to remove excess reagent from the chromatogram before recording the chromatogram zones. This is best done by dipping the treated chromatogram into several fresh 0.5% acetic acid solutions or by „destaining“ for two to two and a half minutes (diffusion destaining apparatus, cf. Fig. 24). The chromatogram is then dried in a stream of cold air for 30 min. (Warm air causes fading of the chromatogram zones!)

This procedure yielded a colorless background, on which the colors of the alkaloid zones became pale brown (narceine), blue (morphine) or violet (codeine, papaverine, narcotine) (Fig. 2B).

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 540$ nm (Fig. 3). The detection limits in substance per chromatogram zone were 20 ng for thebaine and papaverine, 200 ng for codeine, 300 ng for morphine and 500 ng for narceine.

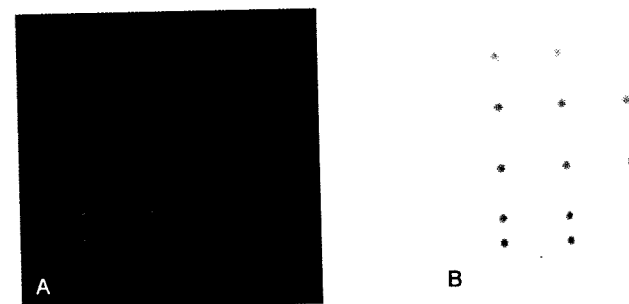


Fig 2: Chromatograms of opium alkaloids (A) after immersion in the reagent solution and (B) after additional washing away of any excess reagent.

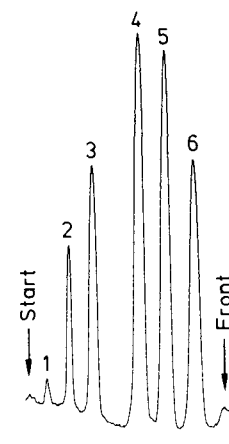


Fig 3: Reflectance scan of a chromatogram track with 1 µg each per chromatogram zone of narceine (1), morphine (2), codeine (3), thebaine (4), papaverine (5) and narcotine (6).

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Potassium Hydroxide Reagent

Reagent for:

- Cumarin glycosides and their aglycones [1, 2]
e.g. umckalin, scopoletin [3]
- Anthraquinone glycosides and their aglycones [2, 4–8]
e.g. chrysophanol, emodin, aloe emodin, rhein [9–12]
physcione [9, 11, 12], alizarin [10]
- Xanthone glycosides and their aglycones [13]
e.g. gentisin, isogentisin [14]
- Dalbergion-glycosides and their aglycones [15]
- Pesticides
e.g. thiophosphate pesticides [16]
such as bayrusil, fospirate, noltran, menazon
maretin, dursban, cythioate
- Nitroaryl esters
e.g. mono and dinitrophenylacetates [17]
- Acetylcholine, adenochochrome, nicotinamide [18]
- Cytostatics (antineoplastic agents)
e.g. 1-ethoxycarbonyl-2-arylazo-2-nitroethane derivatives [19]
- Block copolymers
e.g. polyacrylonitrile [20]
- Dinitrophenylhydrazones
e.g. of pyruvic acid, glycolaldehyde [21]

KOH

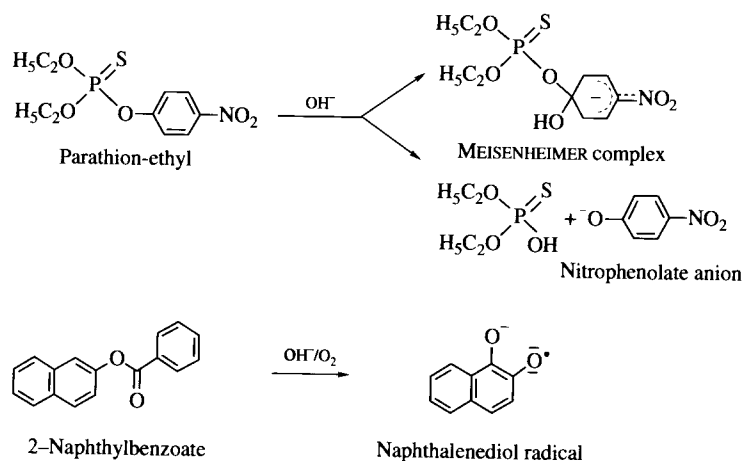
 $M_r = 56.11$

Preparation of the Reagent

Dipping solution	Dissolve 2 g potassium hydroxide in 5 ml water and make up to 50 ml with ethanol [22].
Spray solution	Dissolve 3 to 10 g potassium hydroxide in 100 ml methanol [1, 2, 6, 8–10, 14, 19], ethanol [7, 15, 19] or water [16, 18, 20].
Storage	The reagent solutions can be stored for longer periods.
Substances	Potassium hydroxide pellets Methanol Ethanol

Reaction

The reaction course has not been elucidated (cf. also sodium hydroxide reagent). Hydrolyzation reactions and aromatizations are probably primarily responsible for the formation of colored and fluorescent derivatives. Substituted nitrophenols – e.g. the thiophosphate insecticides – can probably be hydrolyzed to yellow-colored nitrophenolate anions by sodium hydroxide or possibly react to yield yellow MEISENHEIMER complexes. Naphthol derivatives with a tendency to form radicals, e.g. 2-naphthyl benzoate, react with hydrolysis to yield violet-colored mesomerically stabilized 1,2-naphthalenediol radicals.



Method

The chromatograms are dried in a stream of cold air, first sprayed homogeneously with the reagent and, as in the case of anthraquinone derivatives, they are then dried in steam of warm air for a few minutes [10] or at room temperature for 20 min [11]. In the case of pesticides the chromatogram is covered by a glass plate and heated 100–200°C for up to 30 min [16].

Dalbergion glycosides produce green to violet [15], 2-arylo-2-nitroethane derivatives [19] and polyacrylonitrile (20) yellow to orange-red, dinitrophenylhydrazones yellow to purple-brown and anthraquinone derivatives orange-yellow to purple-color substance zones [5, 8, 10, 12] that usually emit yellow or pale red to violet fluorescence in UV light ($\lambda = 254$ or 365 nm) [4, 8, 9]. Some thiophosphate pesticides can also be stimulated to fluorescence with long-wavelength UV light ($\lambda = 365$ nm) [16]. Gentis fluoresces yellow-green [14], cumarins green to dark blue [1].

Note: Methanolic sodium hydroxide solution can replace potassium hydroxide solution [16] (q.v.). The production of color tones and fluorescence is very dependent on the duration and temperature of heating; hence optimal conditions must be determined empirically [16]. Some thiophosphate insecticides do not form fluorescent derivatives [16]. The natural fluorescence of various coumarin derivatives is intensified [1].

It is recommended that the chromatogram treated with reagent be stored for 15 min to allow stabilization of color when undertaking direct quantitation of anthraquinones [10].

The detection limits as substance per chromatogram zone are 1 µg for polyacrylonitrile [20] and 2-arylo-2-nitroethane derivatives [19] and 6–100 ng for thiophosphate pesticides [16].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 500 cellulose and polyamide layers as well as on mixed cellulose/polyamide layers [1].

Procedure Tested 1

Thiophosphate Insecticides [22]

Method

Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer	HPTLC plates Silica gel 60 F ₂₅₄ extra thin layer (MERCK), that were prewashed by dipping in 2-propanol overnight and then activated at 110 °C for 30 min.
Mobile phase	<i>n</i> -Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (909+40+26+25+1).
Migration distance	8 cm
Running time	30 min

Detection and result: The chromatogram was dried for ca. 3 min in a stream of warm air, immersed in the reagent solution for 3 s and then heated to 110 °C for 15 min.

Parathion-methyl (*hR_f* 40–45), fenitrothion (*hR_f* 45–50) and parathion-ethyl (*hR_f* 60–65) appeared as yellow chromatogram zones on a colorless background. The detection limits lay between 6 ng (parathion, parathion-methyl) and 10 ng (fenitrothion) per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 406$ nm (Fig. 1).

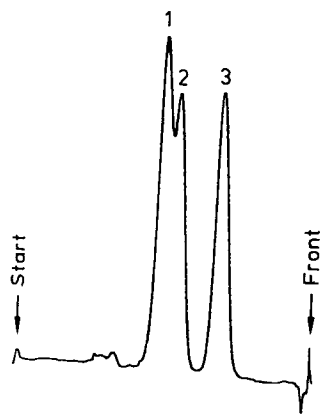


Fig 1: Reflectance scan of a chromatogram track with 300 ng substance per chromatogram zone of parathion-methyl = 1, fenitrothion = 2, parathion-ethyl = 3.

Procedure Tested 2

Cumarin in *Asperulae Herba* (Woodruff) [23]

Sample solution	A 3 g sample of woodruff was added to 30 ml warm methanol and placed in the ultrasonic bath for 10 min. After filtration the solution was concentrated to ca. 20% of the initial volume under reduced pressure. A portion of the solution was centrifuged at 1200 rpm for 2 min and the clear supernatant was applied to the layer as a band.
Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene – <i>tert</i> -butyl methyl ether – acetic acid (10%) (40+40+40). The mobile phase is always freshly made up. This is done by mixing the three mobile phase components in a separating funnel and shaking vigorously several times; the top phase is used as mobile phase.
Migration distance	10 cm
Running time	20 min

Detection and result: The chromatogram was dried in a stream of warm air and then inspected under UV light. Cumarin (*hR_f* 55–60) exhibited fluorescence quenching under short-wavelength UV light ($\lambda = 254$ nm; Fig. 2A); it is not excited to fluorescence emission under long-wavelength UV light ($\lambda = 365$ nm; Fig. 2B). After treatment with the spray solution (1 g KOH pellets in 20 ml ethanol) the cumarin zone produced an intense yellow-green fluorescence when observed under long-wavelength UV light (Fig. 2C), which changed to pale blue after heating the chromatogram (2 min, 100 °C) (Fig. 2D).

It is possible to detect 2 ng cumarin per chromatogram zone visually.

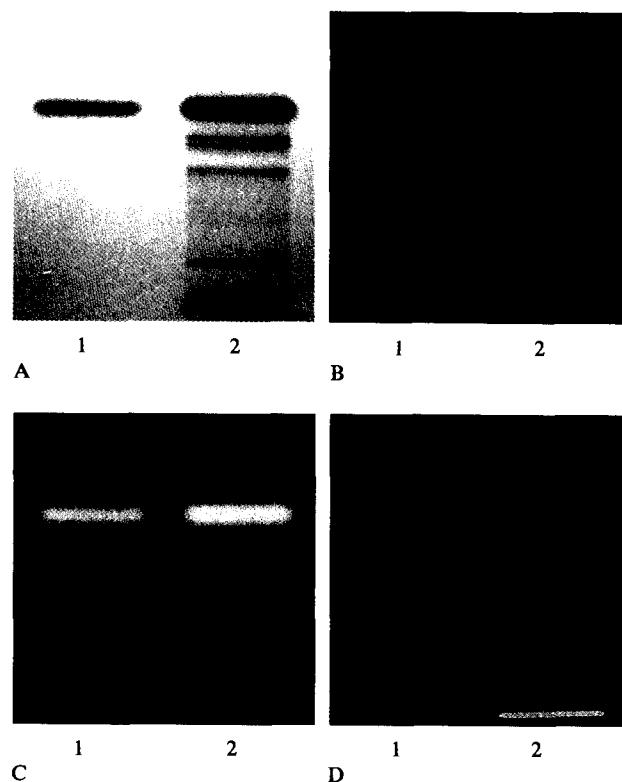


Fig 2: Chromatogram of woodruff extract (track 1: 10 µl 0.1% cumarin solution in methanol, track 2: 100 µl extract; band length 3 cm in each case). Examination in short-wavelength UV light before spraying with the reagent (A) and in long-wavelength UV light before (B) and after spraying (C) and finally after heating (D).

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Preparation of the Reagent

- Dipping solution** Dissolve 2.8 g potassium iodide and 1.4 g soluble starch according to ZULKOWSKY in 70 ml water and dilute with 30 ml absolute ethanol [10].
- Spray solution I** *For peroxides*
Solution A: Mix 10 ml 4% aqueous potassium iodide with 40 ml glacial acetic acid and add a spatula-tip of zinc powder. Filter off the zinc powder immediately before using the spray solution [1, 4].
Solution B: Dissolve 1 g soluble starch in 100 ml water with boiling [1, 4].
- Spray solution II** *For substances containing bromine:* Dissolve 4 g potassium iodide and 2 g soluble starch in 100 ml water with warming [7].

Potassium Iodide–Starch Reagent

Reagent for:

- Peroxides, hydroperoxides [1, 2]
 e. g. (photo)-oxidation products
 of limonene [3]
 linoleic acid [4], methyl linoleate [5]
 methyl oleate and methyl elaidate [6]
- Bromine-containing barbiturates and ureides [7]
- Sulfoxides [8]

under the influence of UV light — to iodine, which reacts with starch to yield the well-known intense blue starch-iodine inclusion complex.

Method

For peroxides: The chromatograms are dried in a stream of warm air and immersed in the dipping solution for 2 s [10]. Alternatively they can first be sprayed homogeneously with spray reagent 1A, allowed to stand for 5 min and then sprayed with spray solution 1B until they are transparent [1, 4].

For substances containing bromine: The dried chromatograms are immersed in the dipping solution for 2 s or sprayed homogeneously with spray solution II and then, while still moist, they are irradiated with intense UV light for ca. 1 to 3 min. [7, 10].

For sulfoxides: The dried chromatograms are sprayed homogeneously with spray reagent III. After a few minutes they can then be sprayed with spray solution IB to increase the color contrast [8, 9].

In all cases intense blue or brown-colored chromatogram zones are produced on a colorless to brownish background.

Potassium Iodide–Starch Reagent 37

Spray solution III *For sulfoxides:* Dissolve 5 g starch and 0.5 g sodium iodide in 100 ml water with warming. Add 1 ml conc. hydrochloric acid and 10 ml of the solution immediately before use [8].

Storage The reagent solutions may be stored for a few days.

Substances Potassium iodide
 Starch, soluble acc. to ZULKOWSKY
 Acetic acid (100%)
 Zinc powder
 Sodium iodide
 Hydrochloric acid (32%)
 Ethanol

Reaction

Note: Separate potassium iodide and starch solutions can also be used successively [2].

The detection limits for bromureides are 40 to 200 ng substance per chromatogram zone [7, 10]. Bromopride, bromazepam, bromhexine and bromocriptine do not react [10].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, cellulose and NH₂ layers. CN, Diol and polyamide phases are unsuitable.

Procedure Tested

Bromureides [10]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 (MERCK).
Mobile phase	Dichloromethane – diethyl ether (17+3).
Migration distance	5 cm
Running time	5 min

Detection and result: The chromatogram was dried in a stream of warm air and immersed in the dipping solution for 2 s. The excess water drops were then removed from the surface of the layer in a stream of cold air. The chromatogram was then intensively irradiated with UV light (mercury lamp St 41, distance from layer 5 cm).

Bromisoval (hR_f 15–20) yielded dark brown chromatogram zones on a light brown background. Bromopride, bromazepam, bromhexine, bromocriptine, caryophyllene epoxide and rose oxide did not react.

The detection limit of bromisoval is 40 ng substance per chromatogram zone.

In situ quantitation: Quantitative evaluation was not possible.

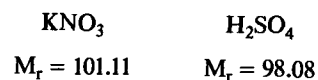
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Potassium Nitrate–Sulfuric Acid Reagent

Reagent for:

- Alkaloids
e.g. colchicine [1]



Preparation of the Reagent

Dipping solution	Dissolve 100 mg potassium nitrate in 5 ml sulfuric acid (95–97%) and make up to 100 ml with ethanol [1].
Storage	The reagent solution can be stored for ca. 1 month.
Substances	Potassium nitrate Sulfuric acid (95–97%) Ethanol

Reaction

The mechanism of the reaction has not been elucidated. Nitration probably takes place.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 4 s or homogeneously sprayed with it, dried in a stream of cold air and then heated to 110 °C for 10 min.

Yellow chromatogram zones are produced on a colorless background.

Note: The detection limit for colchicine is 5 ng substance per chromatogram zone. The reagent can, for example, be used on silica gel, kieselguhr and Si 50000 layer.

Procedure Tested

Colchicine [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that were prewashed before use by developing once to the upper edge of the plate, before application of the sample, with chloroform – methanol (50+50) and then dried at 110 °C for 10 min.
Mobile phase	Acetone – toluene – ammonia solution (25%) (40+15+5).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air for 45 min in order to remove the ammonia completely, then cooled to room temperature (15 min), immersed in the dipping solution for 4 s, dried in a stream of cold air and then heated to 110 °C for 10 min.

Colchicine (R_f 35–40) appeared as a yellow chromatogram zone on a colorless background. The detection limit lay below 5 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation was carried out in reflectance at a wavelength of $\lambda = 380$ nm (Fig. 1).

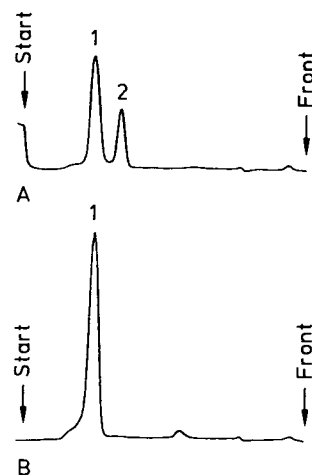


Fig. 1: Reflectance scan of a chromatogram track of a *Colchicum autumnale* extract (A) and of a reference track with 1 µg colchicine (1) per chromatogram zone (B); unknown substance (2).

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Potassium Peroxodisulfate–Silver Nitrate Reagent

Reagent for:

- Aromatic amines and phenols [1–4]
e. g. resorcinol, catechol, aminonaphthols
- Indole, *m*-dinitrobenzene, pyrene [2]
- Sulfapyridine [2, 3]

$K_2S_2O_8$	$AgNO_3$
$M_r = 270.33$	$M_r = 169.87$

Preparation of the Reagent

Dipping solution	Dissolve 1 g potassium peroxodisulfate and 34 mg silver nitrate in 60 ml water and make up to 100 ml with acetone [5].
Spray solution	Dissolve 1 g potassium peroxodisulfate and 17–34 mg silver nitrate in a mixture of 1 ml acetone and 99 ml water [1, 2] or in pure water [3].
Storage	The reagent solutions may be kept for extended periods.
Substances	Potassium peroxodisulfate Silver nitrate Acetone

Reaction

Peroxodisulfate ions oxidize aromatic amines and phenols to colored derivatives, particularly under the catalytic influence of silver ions [1–4].

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and then heated to 50–110 °C for 2–5 min.

Yellow, orange to violet or grey-black chromatogram zones are produced, generally before heating. The background is colorless [1–4].

Note: The dipping solution can also be used as a spray solution. Aromatic amines react more sensitively than do phenols [1]. The presence of acetone in the reagent increases the sensitivity for some substances, e.g. for sulfapyridine [2].

The visual detection limits for aromatic amines and phenols are 100–600 ng substance per chromatogram zone [1–3].

The reagent can, for example, be used on silica gel, kieselguhr and Si 50 000 layers.

Procedure Tested

Aminophenols [5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that were prewashed before use by developing once to the upper edge of the plate, before application of the sample, with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.
Mobile phase	Toluene – methanol (10+10).

Migration distance 5 cm

Running time 15 min

Detection and result: The chromatogram was first dried in a stream of cold air for 5 min, then immersed in the dipping solution for 1 s, briefly dried in a stream of cold air and then heated to 110 °C for 2 min.

2-Aminophenol (hR_f 70–75, λ_{\max} = 430 nm) appeared as a yellow-green chromatogram zone and 4-aminophenol (hR_f 60–65, λ_{\max} = 360–380 nm) as a grey-brown chromatogram zone on a colorless background. The photometric detection limits are 50 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at a mean wavelength of λ = 400 nm (fig. 1).

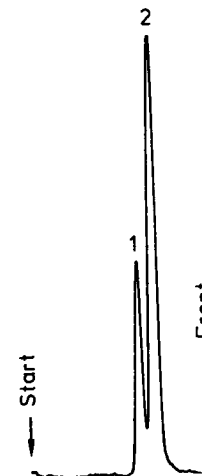


Fig. 1: Absorption scan of a chromatogram track with 200 ng each per chromatogram zone 4-aminophenol (1) and 2-aminophenol (2).

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Selenium Dioxide Reagent

Reagent for:

- Aromatic amines, amino phenols [1]
e.g. aromatic *o*-diamines [2]
- Polyhydric phenols [1]
e.g. resorcinol, pyrogallol
- Reducing substances [1]
e.g. hydrazine, phenylhydrazine, ascorbic acid
- Alkaloids
e.g. brucine, cinchonine [1]



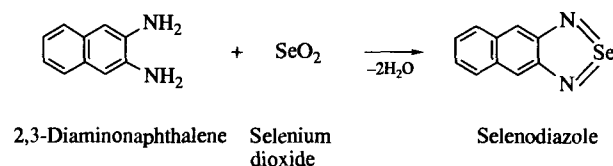
$$M_r = 110.96$$

Preparation of the Reagent

Dipping solution	Dissolve 0.5 g selenium dioxide in 50 ml methanol and add 1 glacial acetic acid [2].
Spray solution	Dissolve 3 g selenium dioxide in 100 ml water [1].
Storage	Both reagents may be stored for longer periods.
Substances	Selenium dioxide Methanol Acetic acid (100%)

Reaction

The reaction mechanism has not been elucidated. Reducing substances presumably release red elementary selenium [1]. Aromatic *o*-diamines yield highly fluorescent selenodiazoles with selenium dioxide.



Method

The chromatograms are freed from mobile phase in a stream of warm air, then immersed twice in the dipping solution for 2 s or sprayed homogeneously with the spray solution and then heated to 120°C for 15–20 min.

Various colored chromatogram zones appear, some before heating, on a colorless background [1]; those produced by aromatic *o*-diamines are excited to fluorescence by long-wavelength UV light ($\lambda = 365$ nm) [2].

Note: Reducing sugars do not react [1]. In the course of a few days the chromatogram zones gradually acquire brown-black discoloration, presumably as a result of the production of elementary selenium [1].

The detection limits for aromatic amines are 1–2 μ g substance per chromatogram zone [1] and 3 ng substance per chromatogram zone for aromatic *o*-diamines [2].

The reagent can be employed, for example, on silica gel, kieselguhr and on Si 50000 layers.

Procedure Tested

Aromatic *o*-Diamines [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	2-Propanol – chlorobenzene – <i>n</i> -hexane – water – ammonium solution (25%) (52+34+20+10+5).
Migration distance	6 cm
Running time	35 min

Detection and result: The chromatogram was freed from mobile phase for 5 min in stream of cold air, immersed twice in the dipping solution for 2 s and then dried for 5 min in a stream of cold air. In order to stabilize and enhance the fluorescence intensity it was then immersed twice for 2 s in a solution of Triton X-100 – chloroform (1+4), with the chromatogram being kept in the dark between and after these dipping processes.

After ca. 30 min, when the chloroform had evaporated, fluorescent chromatogram zones appeared on a dark background on excitation with long-wavelength UV light ($\lambda = 365$ nm): 2,3-diaminonaphthalene (hR_f 70–75), red and 2,3-diaminopyridine (hR_f 55–60), blue. The detection limits were 3 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric scans were carried out at several combinations of excitation and measurement wavelengths (Fig. 1).

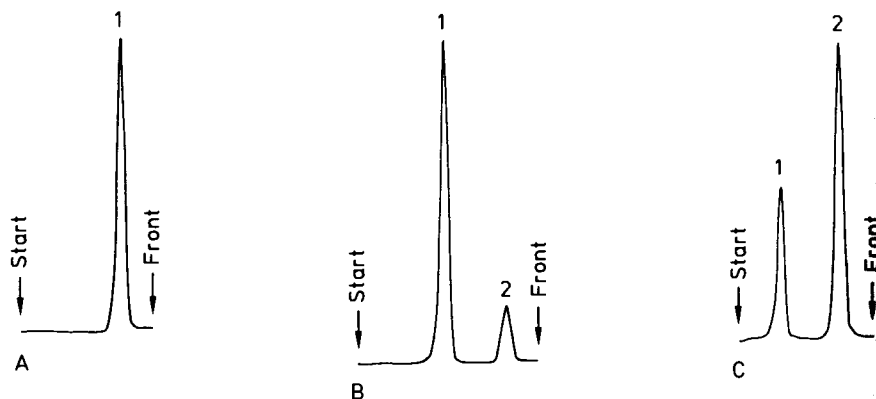


Fig. 1: Fluorescence scans of a chromatogram track with 60 ng each of 2,3-diaminopyridine (1) and 2,3-diaminonaphthalene (2) per chromatogram zone:
 A) $\lambda_{\text{exc}} = 365 \text{ nm}$ and $\lambda_{\text{fl}} > 560 \text{ nm}$ (cut off filter FI 56); B) $\lambda_{\text{exc}} = 313 \text{ nm}$ and $\lambda_{\text{fl}} > 390 \text{ nm}$ (cut off filter FI 39); C) $\lambda_{\text{exc}} = 365 \text{ nm}$ and $\lambda_{\text{fl}} > 390 \text{ nm}$ (cut off filter FI 39).

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Sodium Hydroxide Reagent

Reagent for:

- α -Pyrone derivatives
 - e. g. umbelliprenine [1]
 - cumarin [2–4]
 - dicumarol [5]
- Phenyl-1,3-indandione metabolites [5]
- Mycotoxins
 - e. g. patulin as 2,4-DNPH derivative [6]
- Quinones [7]
 - e. g. antibiotics
 - such as xanthomegnin, viomellein [8]
- Pesticides
 - e. g. insecticides
 - such as phosalone [9], carbofuran [10], sevin [11]
 - organophosphorus pesticides
 - such as bayrusil, cythioate, dursban, menazon [12];
 - paraaxon [13]
 - 2-sec-butyl-4,6-dinitrophenyl herbicides and acaricides [14]
 - such as dinobuton, 2-sec-butyl-4,6-dinitrophenol
- Pharmaceuticals
 - e. g. in urine [15]
- Steroid and stilbene derivatives
 - e. g. *trans*-stilbene metabolites [16]
 - Δ^4 -3-ketosteroids [17]
- Glucose-8-methionine [18]
- Homogentisic acid [19]
- Arabinosylcytosine [20]
- Sennosides [21]
- α -Naphthol [11]

NaOH

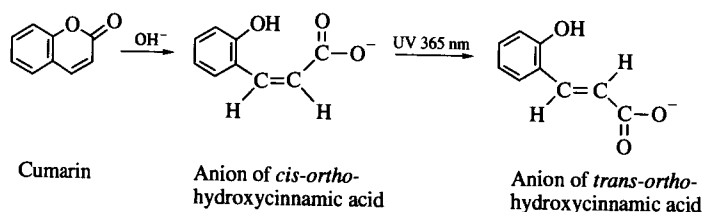
$M_r = 40.00$

Preparation of the Reagent

Dipping solution	Sodium hydroxide solution ($c = 0.1 \dots 1$ mol/L) in methanol-water (60+40) [22].
Spray solution	Sodium hydroxide solution ($c = 1$ to 2.5 mol/L) in methanol-water mixtures (e.g. 50+50) [3, 5-7, 9-14, 16, 18, 21].
Storage	The solutions may be stored in the refrigerator for longer periods.
Substances	Sodium hydroxide pellets Methanol

Reaction

The course of the reaction has not been fully clarified. Hydrolytic and aromatization processes are probably responsible for the formation of colored or fluorescent derivatives (cf. Potassium Hydroxide Reagent). For instance, sevin is converted to α -naphthol [11] and paraoxon to the yellow 4-nitrophenolate anion [13]. In the case of α -pyrone derivatives it is assumed that the alkali metal salt of the *o*-hydroxycinnamic acid produced by hydrolytic cleavage of the pyrone ring is converted from the non-fluorescent *cis*- to the fluorescent *trans*-form by the action of long-wavelength UV light ($\lambda = 365$ nm) [2].



Method

The developed chromatograms are freed from mobile phase in a stream of cold air for 5 min, then immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution and heated to 80–150°C for 5–10 min.

Colored or, under long-wavelength UV light ($\lambda = 365$ nm), yellow or blue fluorescent chromatograph zones appear, even after drying the chromatogram in a stream of cold air, but sometimes only after heating to 80°C [17], to 130°C [9] or 150°C [22] for 5–10 min.

Note: It is occasionally recommended that after it has been sprayed the plate should be covered with a glass plate for several minutes until optimum reaction has occurred [11], or be irradiated with long-wavelength UV light ($\lambda = 365$ nm) [2]. Methanolic potassium hydroxide solution can also be used in place of sodium hydroxide [12] (see Potassium Hydroxide Reagent). The formation of colors and fluorescences depends on the length of heating and on the temperature employed; optimum conditions must be discovered empirically [12].

The 2,4-dinitrophenylhydrazone of patulin and other mono-2,4-dinitrophenylhydrazones form red zones, 2-*sec*-butyl-4-amino-6-nitrophenol appears as a red-orange zone while dinitrophenols and their esters are colored yellow [14]. A whole range of organophosphorus pesticides do not give any reaction [12].

The detection limits per chromatogram zone are 1 ng for sevin and α -naphthol [11], 1 to 100 ng for organophosphorus pesticides, 500 ng for paraoxon [13] and 2 ng for coumarin [3].

The reagent can, for example, be used on silica gel, kieselguhr, Si 50000, NH_2 and diol layers.

Procedure Tested

Δ^4 -3-Ketosteroids and Stilbene Derivatives [22]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Chloroform – methanol (98+2).
Migration distance	8 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed in the dipping solution for 2 s ($c = 1$ mol/L) and heated to 150°C for 10 min.

Weakly fluorescent zones were visible under long-wavelength UV light ($\lambda = 365$ nm) (Fig. 1). Cortisone (hR_f 0–5), dienestrol (hR_f 10–15), 4-androstene-3,17-dione (hR_f 50–55) and 4-cholesten-3-one (hR_f 60–65) had an ochre fluorescence. Diethylstilbestrol (hR_f 10–15), 17 α -ethinyl-1,3,5-estratriene-3,17 β -diol (hR_f 25–30) and estro

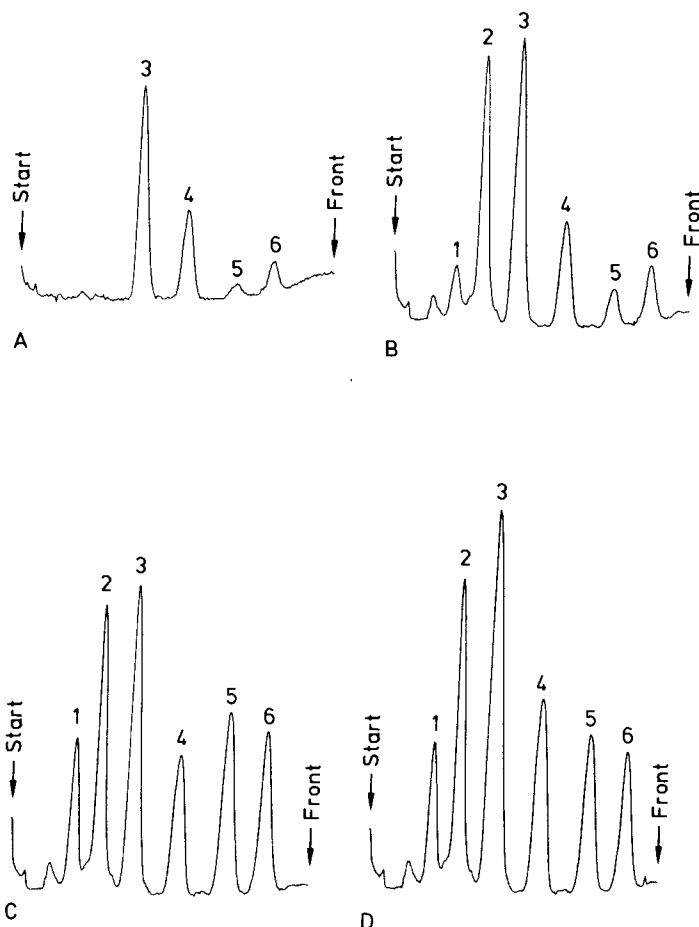


Fig. 1: Fluorescence scan of a chromatogram track with 1 μ g cortisone (1), 100 ng dienestrol (2), 300 ng 17 α -ethinyl-1,3,5-estratriene-3,17 β -diol (3), 100 ng estrone (4) and 1 μ g each of 4-androstene-3,17-dione (5) and 4-cholesten-3-one (6): **A.** before immersion in Triton X-100, **B.** after immersion followed by brief drying, **C.** after heating to 120°C for 10 minutes and **D.** for a further 20 minutes to increase the fluorescence.

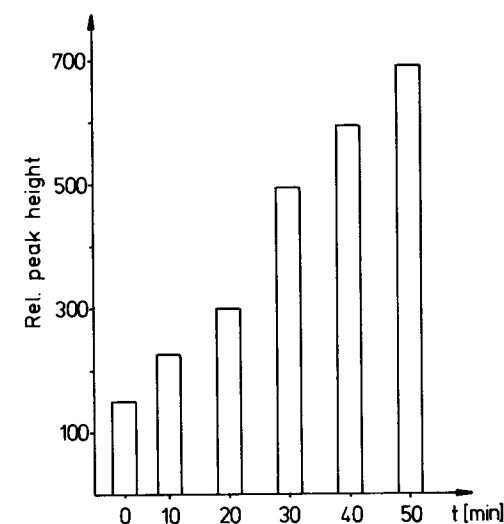


Fig. 2: Increase in the fluorescence intensity of dienestrol as a function of heating time after immersion of the chromatogram in sodium hydroxide solution ($c = 1$ mol/L) followed by treatment with Triton 100-X and heating to 120°C.

After heating the chromatogram was immersed in a solution of 2 ml Triton X-100 in 5 ml chloroform plus 35 ml *n*-hexane in order to intensify the fluorescence and then dried in a stream of cold air. This brought about an appreciable increase in the fluorescence intensity of dienestrol and diethylstilbestrol, while the intensities of the other steroids were only marginally increased (Fig. 1).

Heating the chromatogram to 120°C after treatment with Triton X-100 led to a further increase in sensitivity that was dependent on the length of heating (Fig. 2).

The detection limits lay between 0.5 and 30 ng substance per chromatogram zone

In situ quantitation: The fluorescence scan was carried out at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 430$ nm (cut off filter Fl 43).

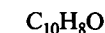
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Sodium Nitrite-Naphthol Reagent

Reagent for:

- Primary aromatic amines [1-3]
e.g. substituted anilines [4, 5]
- Sulfonamides [3, 6]
e.g. sulfadiazine, sulfanilamide, sulfathiazole,
sulfamerazine, sulfamethazine


 $M_r = 69.00$
 $M_r = 144.17$

Sodium nitrite

1-Naphthol

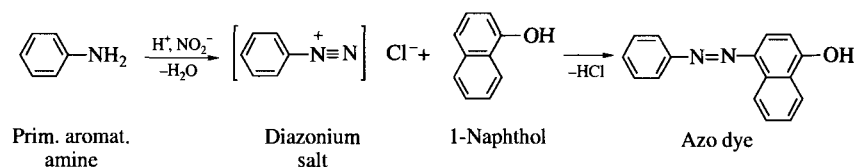
Preparation of the Reagent

- Dipping solution I** Dissolve 5 g sodium nitrite in 20 ml water, add 2 ml hydrochloric acid (32%) and make up to 100 ml with ethanol [7].
- Dipping solution II** Dissolve 5 g 1-naphthol (α -naphthol) in 100 ml ethanol [7].
- Spray solution I** Dissolve 1 to 5 g sodium nitrite in 100 ml hydrochloric acid ($c = 0.2$ to 1 mol/L) [4, 6].
- Spray solution II** Dissolve 0.2 to 5 g 1-naphthol [4] or 2-naphthol (β -naphthol) [5, 6] in 100 ml methanol [4] or sodium hydroxide solution ($c = 0.1 \text{ mol/L}$) [6].
- Storage** All reagent solutions may be stored in the refrigerator for several days [7].
- Substances** Sodium nitrite
1-Naphthol
2-Naphthol

Hydrochloric acid ($c = 1 \text{ mol/L}$)
 Hydrochloric acid (32%)
 Ethanol
 Methanol

Reaction

Primary aromatic amines are first diazotized by the action of sodium nitrite in acidic solution and then coupled, for instance, with 1-naphthol to form azo dyes (cf. BRATTON-MARSHALL reagent, Vol. 1 a).



Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in reagent solution I for 1 s or sprayed with it homogeneously until the layer begins to be transparent, dried for 10 min in a stream of cold air (or 5 min at 50–100 °C [4, 6]), immersed in solution II for 1 s or sprayed with it homogeneously and finally dried in a stream of warm air for 5 min.

Pink-red to orange-colored chromatogram zones are formed, usually at once, on a colorless background. Brown to black colors are also sometimes produced [1].

Note: The diazotization of primary aromatic amines can also be carried out by treating the chromatograms for 3–5 min with nitrous fumes in a twin-trough chamber; the fumes are generated by adding 25% hydrochloric acid to 20% sodium nitrite solution in the free trough [5]. Alternatively the diazotization can be carried out by spraying the chromatograms with a solution of 3% pentyl nitrite and 3% formic acid in diethyl

ether [1]. The α - or β -naphthol in the reagent can be replaced by N-(1-naphthyl)ethylenediamine (cf. BRATTON-MARSHALL reagent, Vol. 1 a).

A few aromatic amines do not react: e.g. *o*-substituted diamines yield benzotriazoles that cannot couple [1].

The detection limits per chromatogram zone are 250 ng for sulfonamides [6] and 80 ng for substituted anilines [7].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Substituted Anilines [7]

Method	Ascending, one-dimensional, two-fold development at 20 °C in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK) that are precleaned by immersion overnight in 2-propanol and then dried at 110 °C for 30 min.
Mobile phase	1. Methanol – acetic acid (100+1). 2. Chloroform – <i>n</i> -hexane – diisopropyl ether – dichloromethane – formic acid (50+35+10+5+0.45).
Migration distance	1. 0.8 cm 2. 6.5 cm
Running time	1. 1 min 2. 25 min

Detection and result: The chromatogram was dried (5 min in a stream of cold air), immersed in dipping solution I for 2 s and then dried in a stream of cold air. It was then immersed in dipping solution II for 1 s and dried in a stream of warm air for 5 min. Sulfanilic acid (hR_f 10–15), 4-isopropylaniline (hR_f 25–30), 4-chloroaniline (hR_f 40–45) and 3,4-dichloroaniline (hR_f 45–50) yielded pink-colored chromatogram zones on a colorless background.

The detection limits lay between 20 ng (4-chloroaniline) and 80 ng (3,4-dichloroaniline) substance per chromatogram zone.

In situ quantitation: The absorption photometric scans were carried out in reflectance at a wavelength of $\lambda = 520 \text{ nm}$ (Fig. 1).

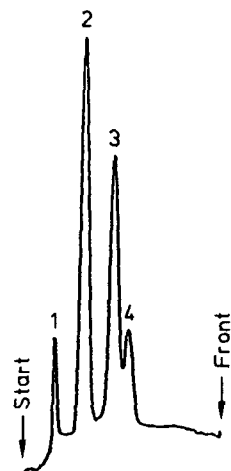


Fig. 1: Reflectance scan of a chromatogram track with 30 ng sulfanilic acid (1) and 100 ng each of 4-isopropylaniline (2), 4-chloroaniline (3) and 3,4-dichloroaniline (4) per chromatogram zone.

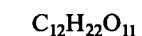
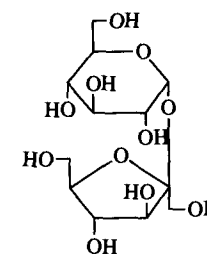
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Sucrose–Hydrochloric Acid Reagent

Reagent for:

- Phenols [1]



$$M_r = 342.30$$

Sucrose



$$M_r = 36.46$$

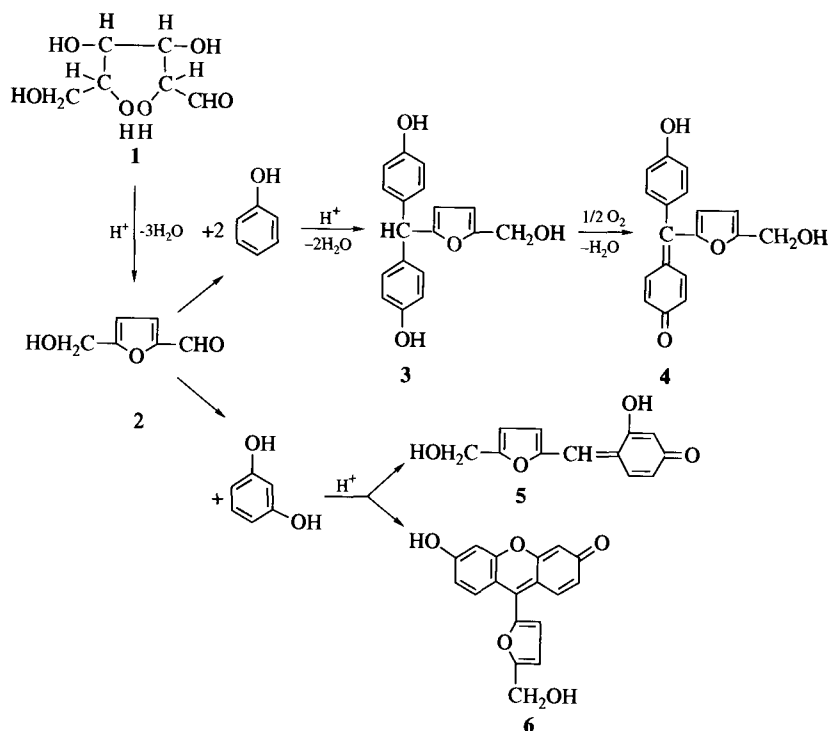
Hydrochloric acid

Preparation of the Reagent

Dipping solution	Dissolve 2 g sucrose in 5 ml water and 5 ml hydrochloric acid (32%) and make up to 100 ml with ethanol [1].
Storage	The dipping solution can be stored over a longer period.
Substances	<p>Sucrose</p> <p>Hydrochloric acid (32%)</p> <p>Ethanol</p> <p>Ammonia solution (25%)</p>

Reaction

The hexoses that are the initial products of acid hydrolysis of sucrose (1) react at elevated temperature under the influence of acids to yield furfural derivatives (2). These condense, for example, with the phenols to yield triarylmethanes (3), these react further by oxidizing to yield colored quinoid derivatives (2, 4). Polyhydric phenols, e.g. resorcinol, on the other hand, yield condensation products of Types 5 and 6 [2].



Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the reagent solution for 2 s or homogeneously sprayed with it and briefly dried in a stream of warm air. It is then heated to 110°C for 2 min and, after cooling to room

temperature, treated with ammonia vapor for 5 min in a twin trough chamber whose empty trough contains 10 ml 25% ammonia solution.

Yellow to brown chromatogram zones are formed on a colorless background.

Note: In long-wavelength UV light ($\lambda = 365$ nm) derivatized phloroglucinol emits a reddish fluorescence that is not suitable for quantitative analysis [1].

The detection limits are in the lower nanogram range.

The reagent can, for example, be used on silica gel, kieselguhr, Si 50000, CN, diol, cellulose and RP layers.

Procedure Tested

“Trihydric” Phenols [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates CN F _{254s} (MERCK).
Mobile phase	Toluene – ethyl acetate (80+20).
Migration distance	6 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air for 10 min, immersed in the reagent solution for 2 s, briefly dried in a stream of warm air and heated to 110°C for 2 min. After cooling to room temperature it was then exposed to ammonia vapors for 5 min (5 ml ammonia solution in the empty trough of a twin trough chamber).

Phloroglucinol (hR_f 15–20) yielded an ochre-colored and pyrogallol (hR_f 35–40) a brown chromatogram zone on a colorless background. The detection limits were 20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric analysis in reflectance was carried out either at the absorption maximum of the pyrogallol derivative at $\lambda_{\max} = 350$ nm (Fig. 1A) or at the absorption maximum of the phloroglucinol derivative at $\lambda_{\max} = 420$ nm (Fig. 1B).

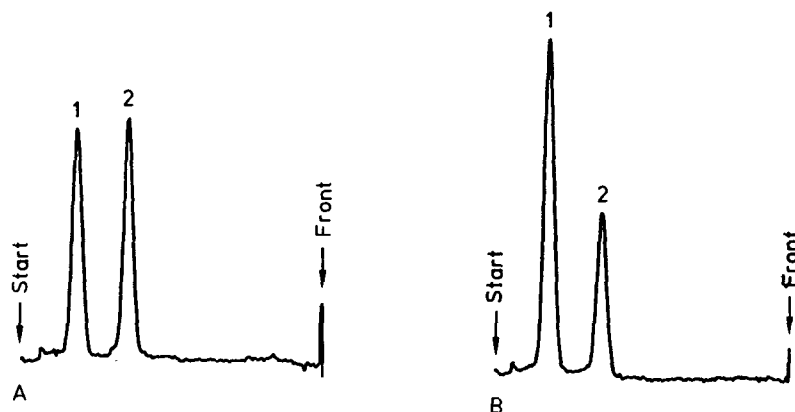


Fig. 1: Reflectance scans of a chromatogram track with 200 ng each of (1) phloroglucinol and (2) pyrogallol per chromatogram zone at wavelengths $\lambda = 350$ nm (A) and $\lambda = 420$ nm (B).

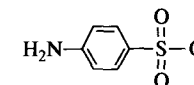
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Sulfanilic Acid, Diazotized Reagent (Pauly's Reagent)

Reagent for:

- Phenols and amines capable of coupling [4, 8]
 - e.g. aniline derivatives, aminophenols [5]
 - such as tyramine [9]
 - phenolcarboxylic acids [1, 3]
 - such as gallic acid, caffeic acid, chlorogenic acid
 - PHB esters [7]
 - phenolic aldehydes
 - such as salicylaldehyde, 4-hydroxybenzaldehyde [3]
 - phenolic alcohols
 - such as coniferyl alcohol [3]
 - chlorophenols [10]
 - naphthylamines [6]
 - flavonoids [3, 27]
 - cumarins [1-3]
- Heterocyclics [4]
 - e.g. imidazole derivatives
 - such as histamine [11], clotrimazole [12]
 - kojic acid [13], imidazole thioethers [14]
 - histidine and metabolites [15]
 - indanedione derivatives [2]
- Proteins
 - e.g. cytochrome C, ovalbumin, aldolase [16]
- Oligo- and polypeptides [18, 21-23, 26]
 - e.g. *Amanita* toxins
 - such as amanitin, phalloidin [17, 20]
 - angiotensin peptides [24, 25]
 - insulin derivatives [19]
- Penicillic acid [13]
- Carboxylic acids [29]
 - e.g. sorbic acid, malic acid, citric acid



$C_6H_7NO_3S$
 $M_r = 173.19$

Solution III	Dissolve 10 g sodium carbonate decahydrate in 100 ml water with cooling in ice. This solution is ready for use after 15 min reaction at 0 °C.
Solution IV	Dissolve 10 g sodium carbonate decahydrate in 100 ml water.
Solution IVa	Dissolve 1.5 g anhydrous sodium carbonate in 15 ml water.
Dipping solution	Mix 10 ml portions of solutions I and IIa with cooling to 4 °C, wait 15 min (4 °C) and treat with 15 ml solution IVa [28].
Spray solution	Mix equal volumes of solutions III and IV immediately before use [4, 8, 20].
Storage	The spray and dipping solutions should always be made up fresh. Solution III is stable for up to 3 days at 0 °C. Solutions I, II and IV are stable over longer periods.
Substances	Sulfanilic acid Hydrochloric acid (32%) Sodium nitrite Sodium carbonate decahydrate Sodium carbonate anhydrous Methanol

Method

The chromatograms are freed from background by spraying with the freshly prepared reagent and are transparent.

Chromatogram zones of various substances are visible immediately but occasionally after longer periods [20].

Note: The sulfanilic acid can also be used for the detection of 2,4,6-Trichlorophenol and 2,3,4-Trichlorophenol; in this case the chromatogram is developed in 10 mol hydrochloric acid) [5]. In the case of the detection of diazotized sulfanilic acid [3]. In the case of the detection of alcoholic iodine solution to cleave sodium carbonate solution and for the detection of 2,4,6-Trichlorophenol and 2,3,4-Trichlorophenol.

The detection limits for phenol are 0.1–1 µg substance per chromatogram.

The reagent can be used, for example, for the detection of phenol on cellulose layers.

Procedure Tested

Nitrophenols [28]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK); before the samples were applied these were immersed for 4 h in 2-propanol and then dried for 30 min at 110 °C. After cooling in the desiccator the layers were protected from atmospheric moisture by covering them 1 cm above the start zones with a glass plate.
Mobile phase	Ethyl acetate — <i>n</i> -hexane (65+35).
Migration distance	6.5 cm
Running time	13 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air (5 min), immersed in the dipping solution for 1 s and then dried for 5 min in a stream of cold air.

2,4-Dinitrophenol (hR_f 0–5, yellow-brown), 2,6-dinitrophenol (hR_f 10–15, yellow-brown), 2,5-dinitrophenol (hR_f 30–35, yellow-brown), 4-nitrophenol (hR_f 40–45, yellow), 3-nitrophenol (hR_f 50–55, light brown) and 2-nitrophenol (hR_f 60–65, pale beige) appeared on a light beige-colored background.

In situ quantitation: The absorption photometric scan in reflectance was carried out at a mean wavelength of $\lambda_{\max} = 420$ nm (Fig. 1). The detection limits per chromatogram zone were 5 ng (2,4- and 2,6-dinitrophenol), 10 ng (2,5-dinitrophenol and 3- and 4-nitrophenol) and 80 ng (2-nitrophenol).

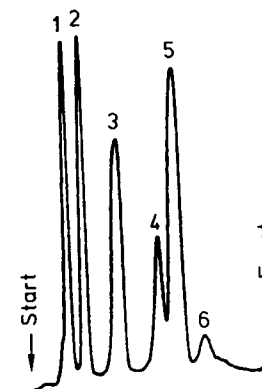


Fig. 1: Reflectance scan of a chromatogram track with 30 ng each of 2,4-dinitrophenol (1) and 2,6-dinitrophenol (2), 60 ng each of 2,5-dinitrophenol (3), 4-nitrophenol (4) and 120 ng each 3-nitrophenol (5) and 2-nitrophenol (6) per chromatogram zone.

References

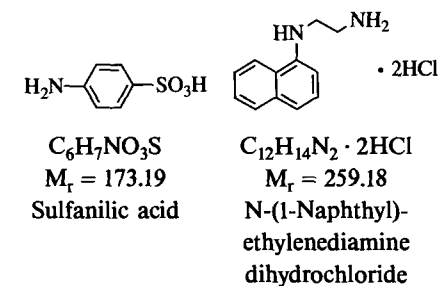
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Sulfanilic Acid–N-(1-Naphthyl)-ethylenediamine Reagent

Reagent for:

- Nitrite [1]
- N-Nitrosamines [2, 3]
- Nitro compounds [4]
 - e.g. explosives
 - such as RDX, HMX, PETN [5]



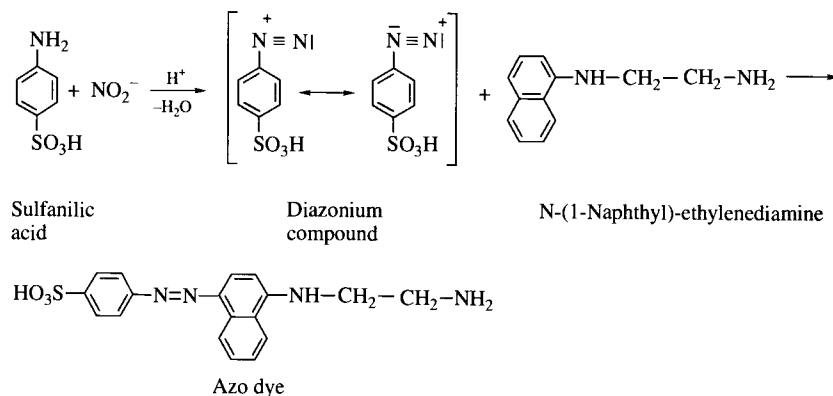
Preparation of the Reagent

- Dipping solution** Dissolve 200 mg sulfanilic acid and 600 mg N-(1-naphthyl)-ethylenediamine dihydrochloride in a mixture of 20 ml hydrochloric acid (32%) and 10 ml water and make up to 200 ml with ethanol (1).
- Storage** The dipping solution may be stored in the refrigerator at 4 °C for ca. 3 weeks.

Substances Sulfanilic acid
N-(1-Naphthyl)-ethylenediamine dihydrochloride
Hydrochloric acid (32%)
Ethanol

Reaction

In the presence of acids, sulfanilic acid — like other primary aromatic amines — reacts with nitrite to yield a diazonium compound that can couple with a suitable aromatic amine to yield an azo dye.



Method

The chromatograms are dried in a stream of warm air for 10 min, then heated to 120°C for 15 min and immersed while still hot (!) in the dipping solution for 1 s or sprayed homogeneously with it and then dried in a stream of cold air for 10 min.

Nitrite, N-nitrosamines and the explosives RDX and HMX yield reddish-violet chromatogram zones on a pale pink-colored background.

Note: Other aromatic amines, e. g. 1- or 2-naphthylamine in acetic acid solution (GRIESS reagent), can be used as coupling agent instead of N-(1-naphthyl)-ethylenediamine

dihydrochloride [2, 3, 5]. In the case of N-nitrosamines the chromatograms should be exposed to bright sunlight for 1–2 h before application of the GRIESS reagent [2], or be irradiated with UV light for 10 min while still moist after application of the reagent [3]. Nitro compounds and explosives are detected by first spraying the chromatogram with sodium hydroxide solution and then treating with the reagent [4, 5].

The detection limit for nitrite is 10 ng substance per chromatogram zone.

The reagent can, for example, be employed on silica gel, kieselguhr and Si 5000 layers.

Procedure Tested

Sodium nitrite [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	2-Butanol — ammonia solution (25 %) (8+2).
Migration distance	4 cm
Running time	25 min

Detection and result: The chromatogram was dried in a stream of warm air (10 min) and then heated to 120°C for 15 min. While still hot (!) it was immersed in the reagent solution (1 s) and then dried in a stream of cold air for 10 min.

Nitrite (hR_F 25–30) yielded a red chromatogram zone on a pale pink background. The detection limit was ca. 10 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 550$ nm (Fig. 1).

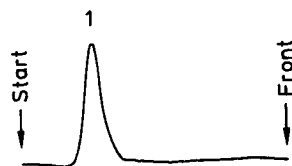


Fig 1: Reflectance scan of a chromatogram track with 50 ng nitrite (1).

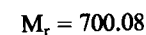
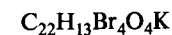
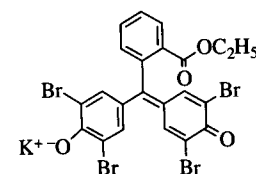
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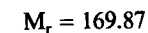
Tetrabromophenolphthalein Ethyl Ester–Silver Nitrate–Citric Acid Reagent (Duggan Reagent)

Reagent for:

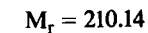
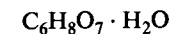
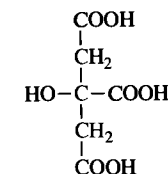
- Thiophosphate pesticides [1]
- Triazines [2]



3,3',5,5'-Tetra-
bromophenolphthalein
ethyl ester K salt



Silver nitrate



Citric acid

Preparation of the Reagent

Dipping solution I Dissolve 0.1 g 3,3',5,5'-tetrabromophenolphthalein ethyl ester potassium salt in 50 ml acetone [2].

Dipping solution II Dissolve 0.5 g silver nitrate in 25 ml water and make up to 100 ml with acetone [2].

Dipping solution III	Dissolve 5 g citric acid monohydrate in 50 ml water and make to 100 ml with actone [2].
Storage	Dipping solutions I and III can be stored in the refrigerator for least 1 week. Dipping solution II should be made up fresh daily
Substances	Tetrabromophenolphthalein ethyl ester potassium salt Silver nitrate Citric acid monohydrate Acetone

Reaction

3,3',5,5'-Tetrabromophthalein ethyl ester potassium salt is a pH indicator that changes from blue to yellow in the pH range 4.2–3. It is known that proteins and alkaloids form blue-colored salt-like adsorption compounds with this indicator that are not destroyed by weak acids [3]. Thiophosphate pesticides and triazines possibly form similar compounds.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in dipping solution I for 3 s, dried in a stream of cold air for 15 min and immersed in dipping solution II for 3 s. After drying in a stream of cold air for 2 min they are immersed in dipping solution III for 3 s and finally dried for 5 min in a stream of cold air.

Blue-colored chromatogram zones are formed on a yellow background.

Note: The dipping solutions can also be used as spray solutions [1]. The chromatogram zones are most intensely blue-colored 5 to 10 min after the chromatograms have been treated with citric acid [1]. The whole layer background then gradually acquires a blue-green coloration over a further 10 min period. However, the difference in color between the chromatogram zones and their background can be restored once more by further treatment with citric acid before the blue color of the derivatives completely and irreversibly fades after about 30 to 40 min [1]. The analogous oxygen compounds to parathion, dichlorvos, naled, mevinphos, phosphamidon and trichlorfon do not react [1].

The detection limits for thiophosphate insecticides are 50–100 ng substance per chromatogram zone [1].

The reagent can, for example, be used on silica gel, kieselguhr, aluminium oxide and Si 50000 layers.

Procedure Tested

Triazines [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK), that were precleaned by dipping in 2-propanol for 12 h (overnight) and then dried at 110°C for 60 min.
Mobile phase	<i>n</i> -Pentane – chloroform – acetonitrile (50+40+10).
Migration distance	7 cm
Running time	25 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air and for 15 min at 60°C, then immersed in dipping solution I for 3 s. After drying for 15 min in a stream of cold air it was immersed in dipping solution II for 3 s, dried for exactly 2 min in a stream of cold air, immersed in dipping solution III for 3 s and finally dried in a stream of cold air for 5 min.

The triazines aziprotryn (*hR_f* 90–95), dipropetryn (*hR_f* 80–85), prometryn (*hR_f* 75–80), ametryn (*hR_f* 65–70), desmetryn (*hR_f* 50–55) and methoprotryn (*hR_f* 40–50) yielded blue-colored chromatogram zones on a yellow background, that turned pale yellowish-green after ca. 1 h. However, this did not interfere with the quantitative evaluation. The detection limit was 20 ng substance per chromatogram zone.

In situ quantitation: The absorption spectrophotometric quantitation was carried out in reflectance at the wavelength $\lambda = 565$ nm (Fig. 1).

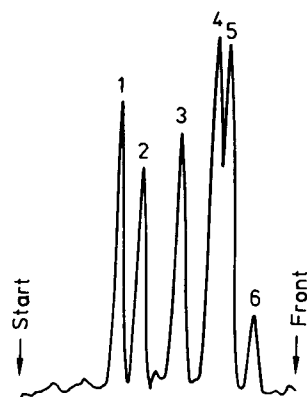


Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone
1 = methoprotryn, 2 = desmetryn, 3 = ametryn, 4 = prometryn, 5 = dipropetryn, 6 = aziprotryn

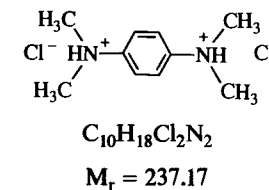
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N,N,N',N'-Tetramethyl-1,4-phenylenediamine Reagent (Wurster's Blue Reagent)

Reagent for:

- Peroxides
e.g. sterol hydroperoxides [1],
such as cholesterol linoleate hydroperoxide [2]
- Steroids [1]
e.g. Δ^4 -3-ketosteroids
- Nitrate esters
e.g. nitroglycerine, diglycerine tetranitrate
ethylene glycol dinitrate [3]
- Polynitroaromatics [3]
- Pesticides and pesticide metabolites
e.g. aniline and urea derivatives [4]
carbamate and organophosphorus insecticides [4]
triazines [4, 5]
- Aromatic amines
e.g. 1,4-phenylenediamine, 2-amino-4-chlorophenol,
4-nitroaniline, 4-amino-3-nitrotoluene [5]

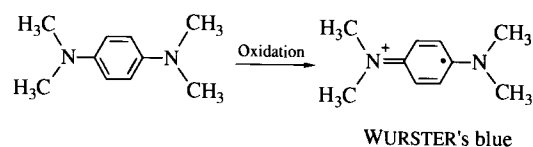


Preparation of the Reagent

Dipping solution	Dissolve 500 mg <i>N,N,N',N'</i> -tetramethyl-1,4-phenylenediammonium dichloride (TPDD) in 100 ml methanol [5] or acetone [3].
Spray solution	Dissolve 1 g TPDD in a mixture of 50 ml methanol, 50 ml water and 1 ml glacial acetic acid [1].
Storage	The dipping solution may be stored in the refrigerator for 1 to 2 weeks. It should always be prepared fresh for quantitative work [5].
Substances	<i>N,N,N',N'</i> -Tetramethyl-1,4-phenylenediammonium dichloride Acetone Methanol Acetic acid (100%)

Reaction

Peroxides oxidize TPDD to WURSTER's blue, a product with a semiquinone diimine structure [1]. Similarly WURSTER's blue is also produced from TPDD by reaction with halogen-containing substances produced by the reaction of aromatic amines and triazines with chlorine gas.



Method

The chromatogram is freed from mobile phase, lightly but homogeneously sprayed with the spray solution or immersed in the dipping solution for 2 s. Triazines and aromatic amines must first be converted to chlorinated derivatives by exposing the chromatogram to chlorine gas (see "Procedure Tested").

After a few minutes drying in air sterol hydroperoxides, nitrate esters, triazines and aromatic amines yield blue-violet chromatogram zones on a pale blue to violet background [1, 3]. Polynitroaromatics yield yellow to dark beige zones [3].

Note: The substances that will react with TPDD reagent do not all react with the chemically related *N,N*-dimethyl-1,4-phenylenediamine reagent (*N,N*-DPDD, q.v.). Hence is possible to use both reagents at the same time to allow differentiation between substances (cf. Table 1) [4]. The reaction on the reagent-treated layer can be accelerated by brief exposure to UV light [3]. When allowed to stand the spray solution relatively rapidly discolours to a dark violet [1], so it should always be made up freshly. The contrast between the colored zones and the background can be improved by warming the chromatogram. The colors of the chromatogram zones remain stable for several days, while the background gradually darkens [1].

The detection limits per chromatogram zone are 50 ng substance for sterol peroxides [1], 20–100 ng for nitrate esters [3] and 5–25 ng for aromatic amines [5].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr or Si 50000 layers.

Table 1: Comparison of the reaction of pesticides (amounts applied 0.8 µg, without chromatographic development) with *N,N*-DPDD (WURSTER's Red) and TPDD (WURSTER's Blue) reagent [4]: – = negative, (+) = weakly positive and +++ = positive reaction.

Substance class/Substance	WURSTER's Red Reaction	WURSTER's Blue Reaction
Aniline derivatives		
Alachlor	–	–
Pendimethalin	–	–
Trifluralin	–	–
Metazachlor	–	(+)
Metolachlor	–	(+)
Carbamate pesticides		
Aldicarb	–	+++
Carbetamide	–	+++
Carbofuran	–	+++
Chloroprotham (CICP)	–	+++
Phenmedipham	–	+++
Protham	–	+++
Urea pesticides		
Chloroxuron	(+)	+++
Chlortoluron	(+)	+++
Diuron	(+)	+++
Dimefuron	–	+++

Table 1 (continued)

Substance class/Substance	WURSTER's Red Reaction	WURSTER's Blue Reaction
Fenuron	—	+++
Isoproturon	—	+++
Linuron	—	+++
Methabenzthiazuron	—	+++
Metobromuron	—	+++
Metoxuron	—	+++
Monolinuron	(+)	+++
Monuron	(+)	+++
Chlorinated hydrocarbons		
Endosulfan	—	—
Organophosphorus pesticides		
Azinophos-ethyl	—	+++
Chlorphenvinfos	—	—
Parathion-ethyl	—	(+)
Parathion-methyl	—	(+)
Triazines		
Atrazine	+++	+++
Cyanazine	+++	+++
Desisopropylatrazine	+++	+++
Hexazinon	—	(+)
Metamitron	—	—
Metribuzin	(+)	—
Prometryn	+++	+++
Propazine	+++	+++
Sebutylazine	+++	+++
Simazine	+++	+++
Terbutryn	+++	+++
Terbutylazine	+++	+++
Uracil derivatives		
Bromacil	—	+++
Aromatic nitro compounds		
Dinocap	—	—
Dinoseb acetate	—	—
Miscellaneous pesticides		
Amitrole	—	+++
Bentazon	—	+++
Chloridazon	—	+++
Crimidine	—	(+)
Vinclozolin	—	+++

Table 1 (continued)

Substance class/Substance	WURSTER's Red Reaction	WURSTER's Blue Reaction
Pesticide metabolites		
Aniline	—	+++
4-Bromoaniline	+++	+++
3-Chloroaniline	+++	+++
4-Chloroaniline	+++	+++
4-Chlorophenol	—	+++
Desethylatrazine	—	+++
Dichlobenil	—	—
3,4-Dichloroaniline	+++	+++
2,4-Dichlorophenol	+++	+++
2,6-Dimethylaniline	—	+++

Procedure Tested

Aromatic Amines [5]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Toluene — ethanol (19 + 1).
Migration distance	8 cm
Running time	13 min

Detection and result: The chromatogram was first dried in a stream of cold air for 10 min, it was then placed for 2 min in a chamber filled with chlorine gas (cylinder, then freed from excess chlorine in a stream of cold air for **exactly** 5 min (fume cup board!)) and immersed in the dipping solution for 2 s.

After drying for 10 min in a stream of cold air 1,4-phenylenediamine (hR_f 5–10), 2-amino-4-chlorophenol (hR_f 15–20), 4-nitroaniline (hR_f 25–30) and 1,4-amino-3-nitro toluene (hR_f 50–55) appeared as blue-violet chromatogram zones on a blue background. These could be recognized without difficulty for several days from the back of the chromatogram.

The detection limits per chromatogram zone lay between 5 ng (1,4-phenylenediamine, 4-nitroaniline) and 25 ng (2-amino-4-chlorophenol, 4-amino-3-nitrotoluene).

In situ quantitation: The photometric evaluation was carried out in reflectance at a wavelength of $\lambda = 608 \text{ nm}$ (Fig. 1).

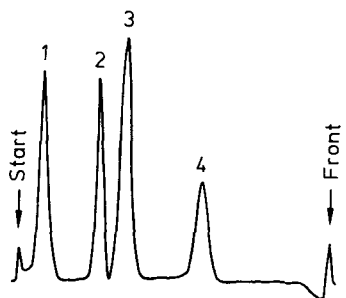


Fig. 1: Reflectance scan of a chromatogram track with 125 ng each substance per chromatogram zone: 1 = 1,4-phenylenediamine, 2 = 2-amino-4-chlorophenol, 3 = 4-nitroaniline, 4 = 4-amino-3-nitrotoluene.

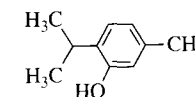
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Thymol–Sulfuric Acid Reagent

Reagent for:

- Sugars
e.g. mono-, di- and trisaccharides [1–10, 13]
fructo-oligosaccharides [1]
- Polyglycerols [11]
- Sugar alcohols
e.g. sorbitol [12]
- Uronic acids [2, 4, 6]



$\text{C}_{10}\text{H}_{14}\text{O}$

$M_r = 150.22$

Thymol

H_2SO_4

$M_r = 98.08$

Sulfuric acid

Preparation of the Reagent

Dipping solution	Dissolve 0.5 g thymol in 95 ml ethanol (96 %) and cautiously 5 ml conc. sulfuric acid.
Storage	The reagent solution may be kept for about 2 days [1].
Substances	Thymol Ethanol (96 %) Sulfuric acid (95–97 %)

Reaction

The mechanism has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in the reagent solution for 3 s [1] or sprayed homogeneously with it until the layers begin to become transparent [2, 11, 13]. They are then heated to 110–125 °C for 5 to 20 min [1, 2, 7, 10, 11, 13] or, in the case of sorbitol, to 170 °C for 10–15 min [12].

The sugars appear as chromatogram zones of various colors (yellow, pale pink, red to blue) on an almost colorless background [1–3, 6, 7]. Uronic acids acquire a beige to violet-pink coloration [2, 4, 6].

Note: The various tones of the colors produced by the sugars, that alter during the first hour after reaction [1, 2], make it possible to differentiate according to color. Glucose and galactose are somewhat less strongly pigmented than the other sugars [1]. Prolongation of the heating after dipping or spraying leads to the fading of some zones at least on silica gel layers impregnated with bisulfite [10].

The detection limits for sugars are of the order of 25 ng substance per chromatogram zone [1].

The reagent can, for example, be used on silica gel, sodium acetate, bisulfite- or boric acid-impregnated silica gel layers, kieselguhr and silica gel/kieselguhr layers.

Procedure Tested

Fructo-Oligosaccharides [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates or TLC aluminium sheets silica gel 60 F ₂₅₄ (MERCK).

Mobile phase	Ethyl acetate – ethanol (96%) – acetic acid (60%) – boric acid solution (cold saturated) (50+20+10+10).
Migration distance	10 cm
Running time	50–55 min

Detection and result: The chromatogram was dried at 55 °C for 30 min, immersed in the reagent solution for 3 s after cooling and then heated to 110 °C for 10 min.

The sugars fructosyl-nystose (hR_f 15–20), maltotetraose (hR_f 15–20), nystose (hR_f 20), 1-kestose (hR_f 20–25), 6-kestose (hR_f 20–25), raffinose (hR_f 20–25), melibiose (hR_f 20–25), maltotriose (hR_f 20–25), panose (hR_f 20–25), melezitose (hR_f 25), lactose (hR_f 25), sorbose (hR_f 25–30), trehalose (hR_f 25–30), neokestose (hR_f 25–30), turanose (hR_f 25–30), fructose (hR_f 30), maltose (hR_f 30), sucrose (hR_f 30–35), galactose (hR_f 35–40), glucose (hR_f 40), arabinose (hR_f 40–45), mannose (hR_f 40–45), xylose (hR_f 45–50) and ribose (hR_f 50) appeared as reddish-blue chromatogram zones on a pale background (Fig. 1A).

In situ quantitation: The photometric measurement in reflectance was carried out at $\lambda = 525$ nm (Fig. 1B). In order to ensure that the zone coloration had stabilized, scanning was not commenced until ca. 30 min after the dipping process. The detection limit for sugars was of the order of 25 ng substance per chromatogram zone.

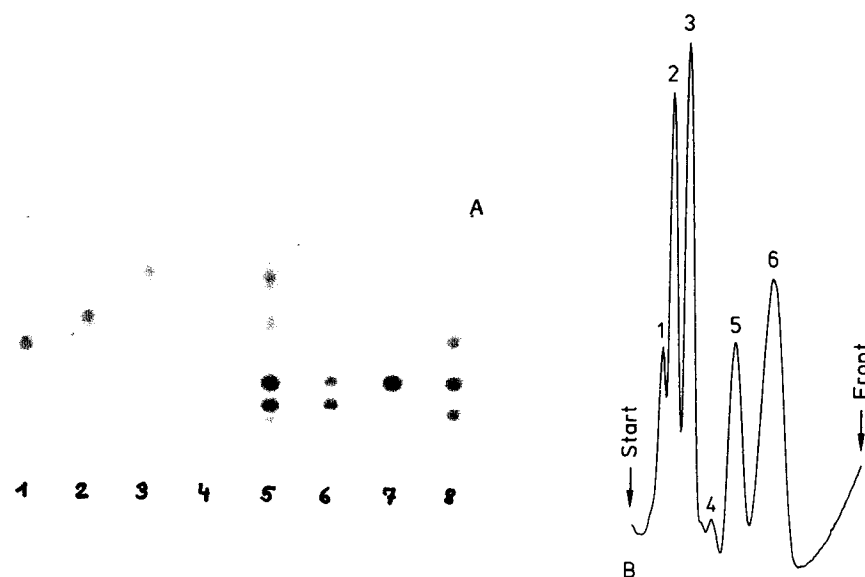


Fig. 1: (A) Chromatographic separation of sugars. Track 1: fructose, 2: sucrose, 3: glucose, 4: mixture of the substances in tracks 1-3, 5: mixture of substances in tracks 1-3 and 6, 6: Fructo-oligosaccharides, 7: 1-kestose, 8: mixture of glucose, maltose, maltotriose and maltotetraose. (B) Absorption scan of track 5 with 200 ng each substance per chromatogram zone: 1 = fructosyl-nystose, 2 = nystose, 3 = 1-kestose, 4 = fructose, 5 = sucrose, 6 = glucose.

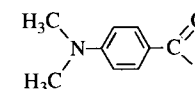
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Tin(II) Chloride-Hydrochloric Acid-4-(Dimethylamino)-benzaldehyde Reagent

Reagent for:

- Aromatic nitrocompounds [1]
- e. g. nitrophenols [2, 3]
- pesticides [2]
- carboxylic acid 2,4-dinitrobenzylesters [4]



$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	HCl	$\text{C}_9\text{H}_{11}\text{NO}$
$M_r = 255.63$	$M_r = 36.46$	$M_r = 149.19$
Tin(II) chloride	Hydrochloric acid	4-(Dimethylamino)-benzaldehyde

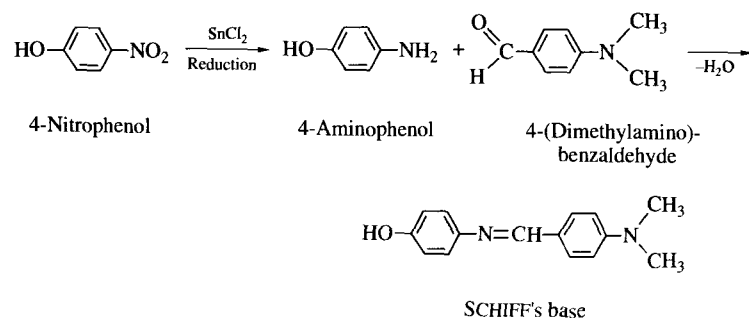
Preparation of the Reagent

- Dipping solution I** Dissolve 2 g tin(II) chloride dihydrate in a mixture of 20 ml hydrochloric acid (32%) and 30 ml methanol [3].
- Dipping solution II** Dissolve 0.25 g 4-(dimethylamino)-benzaldehyde in 50 ml of mixture of ethanol and 1-butanol (50+50) [3].
- Spray solution I** Treat 3 ml 15 percent aqueous tin(II) chloride solution with 15 hydrochloric acid (32%) and dilute with 180 ml water [1, 2].

Spray solution II	Dissolve 1 g 4-(dimethylamino)-benzaldehyde in a mixture of 30 ml ethanol, 3 ml hydrochloric acid (32%) and 180 ml 1-butanol [1, 2].
Storage	The reagent solutions should always be made up fresh.
Substances	Tin(II) chloride dihydrate 4-(dimethylamino)-benzaldehyde Hydrochloric acid (32%) Ethanol Methanol 1-Butanol

Reaction

Tin(II) chloride reduces aromatic nitro compounds to the corresponding amines, these then react with 4-(dimethylamino)-benzaldehyde to yield colored SCHIFF's bases.



Method

The developed chromatograms are dried in a stream of warm air, immersed in dipping solution I for 1 s or sprayed homogeneously with spray solution I and heated to 105–110 °C for 10 min. After cooling the plates are immersed in dipping solution II for 1 s or sprayed homogeneously with spray solution II and dried for 5 min in a stream of cold air.

Chromatogram zones of various colors are produced on an almost colorless background, most of them can be excited to emit fluorescent light on exposure to long-wavelength UV light ($\lambda = 365 \text{ nm}$).

Note: The aromatic amines produced by reduction with SnCl_2 in acidic medium be detected with fluoescamine (after neutralization of the layer by spraying with dium carbonate) instead of 4-(dimethylamino)-benzaldehyde [5].

In the case of dinitrophenols the detection limits are 100 pg to 200 ng substance chromatogram zone [2, 3].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Nitrophenols [3]

Method	Ascending, one-dimensional step development in a trough chamber with 5 min drying in cold air between the two development steps (1st development at room temperature without and 2nd development at -20°C with chamber saturation).
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK); before application the samples the layers were washed by immersing them for 5 min in 2-propanol, then dried at 110 °C for 30 min, before being applied over silica gel in a desiccator. When the samples were being applied the layer above the application zone was covered with a plate to avoid adsorption of moisture from the atmosphere.
Mobile phase	1. Methanol 2. Ethyl acetate – <i>n</i> -hexane (65+35).
Migration distance	1. 0.7 cm 2. 7 cm
Running time	1. 40 s 2. 20 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air, immersed in dipping solution I for 1 s and then heated to 110 °C for 10 min. After cooling to room temperature, it was immersed in dipping solution II for 1 s.

dried in a stream of cold air for 5 min. Finally the plate was dipped in a solution of liquid paraffin – *n*-hexane (1+2) to stabilize and enhance the fluorescence.

In daylight the nitrophenols appeared as variously colored chromatogram zones on a pale yellow background, under long-wavelength UV light ($\lambda = 365$ nm) they were excited to the emission of fluorescence. The associated hR_f values, colors and fluorescence colors are listed in the table below:

Substance	hR_f	Color	Fluorescence color
2,4-Dinitrophenol	10–15	orange	yellow
2,6-Dinitrophenol	20–25	orange	yellow
2,5-Dinitrophenol	35–40	red	orange
4-Nitrophenol	60–65	yellow	green
3-Nitrophenol	65–70	yellow-green	green
2-Nitrophenol	80–85	yellow	pale green

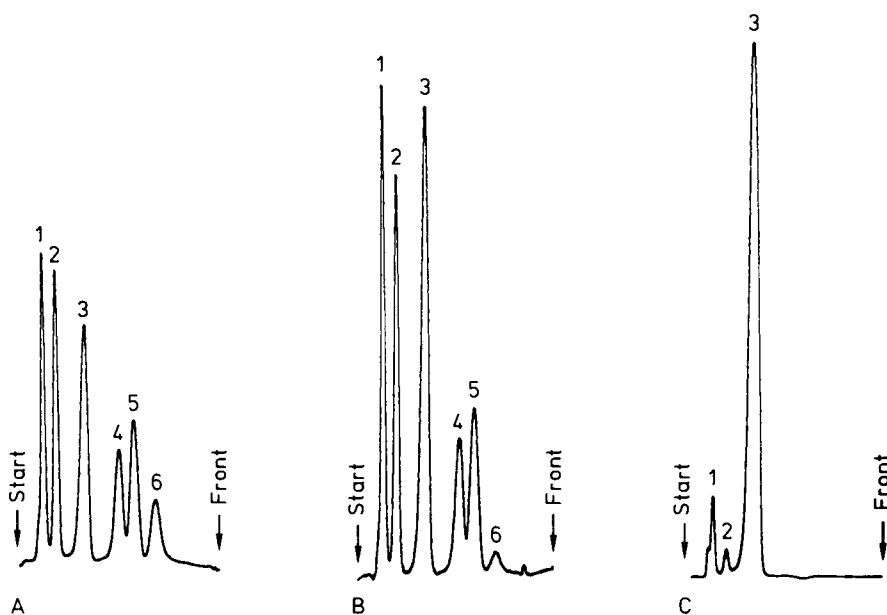


Fig. 1: Reflectance (A) and fluorescence scans (B, C) of a chromatogram track with 30 ng each of 2,4-dinitrophenol (1) and 2,6-dinitrophenol (2), 60 ng each of 2,5-dinitrophenol (3) and 4-nitrophenol (4) and 120 ng each of 3-nitrophenol (5) and 2-nitrophenol (6) per chromatogram zone. Reflectance measurement at $\lambda = 490$ nm (A), fluorescence measurements at $\lambda_{exc} = 408$ nm and $\lambda_{em} > 460$ nm (B) and at $\lambda_{exc} = 546$ nm and $\lambda_{em} > 560$ nm (C).

In situ quantitation: The absorption photometric scan in reflectance was carried out, for example, at a mean wavelength of $\lambda = 490$ nm (Fig. 1). Here the detection limits lie between 100 pg (2,4- and 2,6-dinitrophenol) and 2 ng (2- and 3-nitrophenol). The fluorimetric scan, for example, may be carried out at $\lambda_{exc} = 408$ nm and the fluorescence emission be detected at $\lambda_{em} > 460$ nm (cut off filter FI 46).

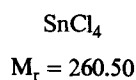
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Tin(IV) Chloride Reagent

Reagent for:

- Sterols, steroids [1, 2]
- Sapogenins [3]
- Triterpenes [2]
- Fatty acids [1]
- Amino acids [1]
- Purines [1]
- Pyrimidines [1]
- Carbohydrates [1]
- Flavonoids [4]
- Phenols, polyphenols [2]



Preparation of the Reagent

Dipping solution	Add 2.5 ml tin(IV) chloride to 40 ml of a mixture of equal volumes chloroform and glacial acetic acid [4].
Storage	The dipping solution may be stored for at least one week.
Substances	Tin(IV) chloride Chloroform Acetic acid (100%)

Reaction

The reaction mechanism has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of cold air and then immersed in the dipping solution for 2 s or sprayed homogeneously with it and then dried for ca. 3 min in a stream of cold air [4].

After reaction the flavonoids, that exhibit weak fluorescence even before derivatization, appear on a colorless background as yellow chromatogram zones; they are excited to yellow to reddish-yellow fluorescence by long-wavelength UV light ($\lambda = 365 \text{ nm}$) [4].

Note: Many substances do not react until the temperature is raised, e. g. to 160–200°C [1]. The reagent can also be applied via the vapor phase at 160°C [1].

The detection limits for flavonoid substances are 5–10 ng substance per chromatogram zone [4].

The reagent can be employed on silica gel, kieselguhr and on Si 50000 layers.

Procedure Tested

Flavonoids [4]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation. After sample application the TLC plates were preconditioned for 30 min at 100% relative humidity and then developed immediately.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Ethyl acetate – water – formic acid (85+15+10).

Migration distance 5 cm

Running time 15 min

Detection and result: The chromatogram was dried for ca. 3 min in a stream of cold air, immersed in the reagent solution for 2 s and then dried for ca. 3 min in a stream of cold air.

The chromatogram zones, that were slightly fluorescent even before derivatization, appeared as yellow zones on a colorless background, under long-wavelength light ($\lambda = 365$ nm) they fluoresced, yellow in the case of rutin (hR_f 20–25), red-yellow in the case of quercitrin (hR_f 60–65) and quercetin (hR_f 85–90).

On account of the noisier background the detection limits for fluorimetric determination were twice as high as those for reflectance determination where they were 5 ng substance per chromatogram zone. An additional immersion in liquid paraffin – *n*-hexane (1+2) did not lead to an intensification of the fluorescence.

In situ quantitation: The absorption photometric scan in reflectance was carried out at $\lambda = 420$ nm (Fig. 1A), and the fluorescence scan was carried out at $\lambda_{\text{exc}} = 436$ nm and $\lambda_{\text{fl}} > 560$ nm (cut off filter F1 56) (Fig. 1B).

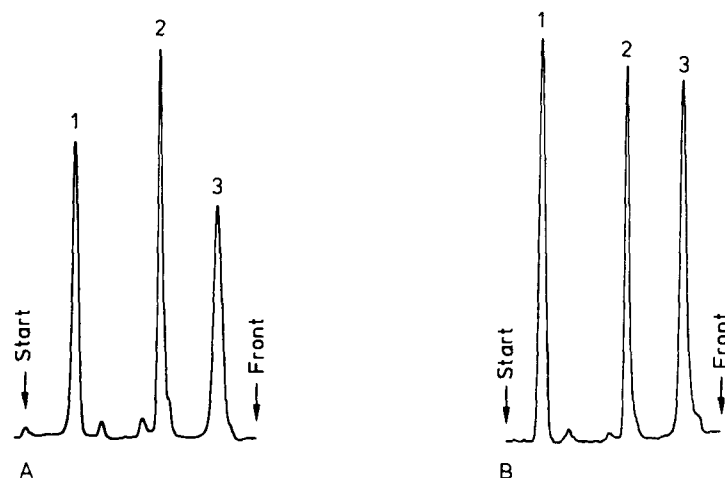


Fig. 1: Reflectance scan (A) and fluorescence scan (B) of a chromatogram track with 200 ng (A) and 100 ng (B) each substance per chromatogram zone: 1 = rutin, 2 = quercitrin, 3 = quercetin.

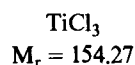
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Titanium(III) Chloride– Hydrochloric Acid Reagent

Reagent for:

- Ascorbic acid, dehydroascorbic acid [1, 2]



Preparation of the Reagent

Dipping solution	Make 15 ml titanium(III) chloride solution (c = 15% in 10 percent hydrochloric acid) up to 100 ml with ethanol (96%) [2].
Storage	The dipping solution can be stored for longer periods.
Substances	Titanium(III) chloride (15% in 10 percent hydrochloric acid) Ethanol (96%)

Reaction

The mechanism of the reaction is unknown.

Method

The dried chromatograms (2 min in a stream of cold air) are immersed in the reagent solution for 10 s or sprayed homogeneously with it and then heated to 110 °C for 10 min.

Yellow chromatogram zones are produced on a colorless background.

Note: The photometric detection limits for ascorbic and dehydroascorbic acids are less than 50 ng substance per chromatogram zone.

The reagent can, for example, be employed on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Ascorbic Acid and Dehydroascorbic Acid [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK).
Mobile phase	Ethanol (96%) – acetic acid (10%) (95+5).
Migration distance	5 cm
Running time	30 min

Detection and result: The dried chromatogram was immersed in the reagent solution for 10 s and then heated to 110 °C for 10 min.

Ascorbic acid (hR_f 50–55) and dehydroascorbic acid (hR_f 65–70) appeared as yellow chromatogram zones on a colorless background.

The detection limits for both ascorbic acid and dehydroascorbic acid were ca. 50 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 400$ nm (Fig. 1).

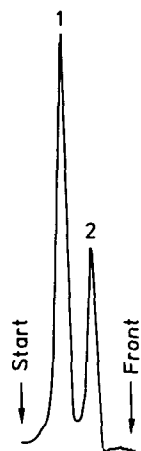


Fig 1: Reflectance scan of a chromatogram track with 2 µg each of ascorbic acid (1) and dehydroascorbic acid (2) per chromatogram zone.

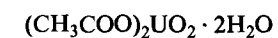
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Uranyl Acetate Reagent

Reagent for:

- Substances absorbing UV light
e.g. aromatics [1, 2], purines [1]
- Histidine [2]
- Sterols, fatty acids, triglycerides,
essential oil components [3]
- Flavones, quinones



$$M_r = 424.15$$

Preparation of the Reagent

Dipping solution	Dissolve 1 g uranyl acetate in 20 ml water with warming and make up to 100 ml with ethanol [3].
Spray solution	Dissolve 1 g uranyl acetate in 100 ml water [1, 2].
Storage	The dipping solution can be stored over a longer period.
Substances	Uranyl acetate dihydrate Ethanol

Reaction

Detection is primarily based on the principle of fluorescence quenching by substances absorbing UV light. It is also possible to detect certain substances whose absorption wavelengths interfere with the uranyl cation [1].

Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and then heated to 100–120 °C for 10–30 min [1].

When examined under UV light ($\lambda = 254$ or 365 nm) dark chromatogram zones are observed on a yellow-green fluorescent background.

Note: Uranyl nitrate can be used instead of uranyl acetate [1]. The detection limits for purines are 10 ng substance per chromatogram zone.

The reagent can, for example, be used on silica gel, kieselguhr and Si 50000 layers; cellulose [1] and RP layers are unsuitable.

Procedure Tested

Sterols, Fatty Acids, Triglycerides [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK). Before application of the samples the layers were washed by immersing them for 1 h in methanol, then dried at 110 °C for 30 min.
Mobile phase	<i>n</i> -Hexane – diethyl ether – glacial acetic acid (80 + 20 + 1).

Migration distance 5 cm

Running time 7 min

Detection and result: The chromatogram was heated to 120 °C for 60 min and the cooled to room temperature, dipped in the reagent solution for 3 s and then dried : 120 °C for 30 min. Cholesterol (hR_f 10–15), stearic acid (hR_f 20–25) and tripalmitin (hR_f 45–50) were visible under long-wavelength UV light ($\lambda = 365$ nm) as dark chromatographic zones on a yellow fluorescent background. The visual detection limits were 100 ng substance per chromatogram zone for cholesterol and tripalmitin and 500 ng substance per chromatogram zone for stearic acid.

In situ quantitation: The fluorimetric analysis was carried out at $\lambda_{exc} = 313$ nm and $\lambda_{fl} > 560$ nm (cut off filter FI 56). The chromatogram zones gave a negative signal (the fluorescent background was set at 100% emission).

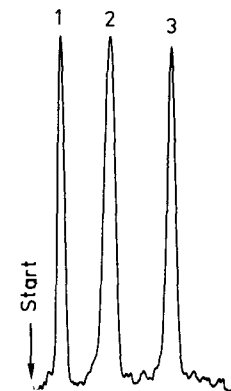


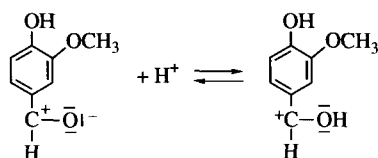
Fig. 1: Fluorescence scan of a chromatogram track with 400 ng cholesterol (1), 200 mg stearic acid (2) and 400 ng tripalmitin (3) per chromatogram zone.

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Vanillin Reagents

The “aldehyde acid” reactions have already been described generally in Chapter 2. There it was pointed out that a nucleophilic attack at a carbonyl group is particularly easy when this is attached to an aromatic ring that bears an “electron withdrawing” group at position 4. The reactivity of the carbonyl group is greatly increased in acid medium:



Classical examples of this type of reaction are the various dimethylaminobenzaldehyde reagents (*q.v.*) and vanillin-acid reagents, of which one, the vanillin-phosphoric acid reagent, is already included in Volume 1a. The aldol condensation of estrogens is an example for the reaction mechanism (cf. Chapter 2, Table 6). According to MALOWAN indole derivatives react in a similar manner [1]. LONGO has postulated that catechins yield intensely colored triphenylmethane dyes [2].

In order to be able to recognize the influences exerted by the various mineral acids used in vanillin reagents the three reagent variants listed below were prepared:

Reagent 1: A solution of 1 g vanillin in 70 ml ethanol (96%) was treated cautiously with 10 ml conc. sulfuric acid.

Reagent 2: A solution of 1 g vanillin in 70 ml ethanol (96%) was treated with 10 ml *ortho*-phosphoric acid (85%).

Reagent 3: A solution of 1 g vanillin in 70 ml ethanol (96%) was treated cautiously with 10 ml fuming hydrochloric acid (37%).

Their reaction was tested on the individual components of the test mixture indole, ergotamine tartrate, ergotamine and ergobasine [ergometrine] [3]. The results obtained were as follows:

- In the case of reagents 1 and 3 the indole zone could be recognized even before heating, while the ergot alkaloids only became visible on heating to 70°C.

- In the case of reagent 2 the zones for indole and the three ergot alkaloids only appeared as strawberry red, or violet zones on a pale background when the plate was heated to 70°C.
- The coloration of the chromatogram zones was the same for all three variants of the reagents and did not depend on the nature of the mineral acid used.
- In the case of reagent 1 the background turned yellow transiently on heating and then returned to a white color on cooling.
- The detection limits obtained are tabulated below:

Substance	Detection limits in ng per chromatogram zone		
	H ₂ SO ₄	H ₃ PO ₄	HCl
Indole	7	6	8
Ergotamine tartrate	25	25	22
Ergotamine	8	14	20
Ergobasine	8	6	20

Evidently the reaction of the indoles investigated with fuming hydrochloric acid is less sensitive as is the case for the two other vanillin reagents.

Aromatic aldehydes react in basic as well as in acidic medium. Thus vanillin and primary amines yield SCHIFF's bases (cf. vanillin-potassium hydroxide reagent in Volume 1a). Colored phenolates are formed at the same time. As would be expected secondary amines, indole derivatives and lysergic acid derivatives do not react.

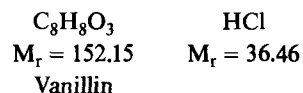
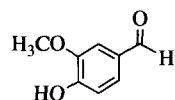
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Vanillin-Hydrochloric Acid Reagent

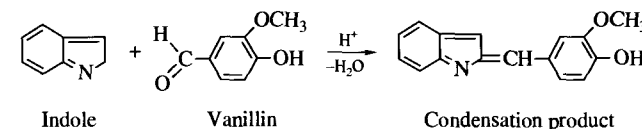
Reagent for:

- Phenols
e.g. hydroxyindole derivatives [1, 2]
- Catechins, gallocatechins [3-12]
- Alkaloids
e.g. lysergic acid derivatives [2]



Reaction

In the presence of strong acids catechins react with aromatic aldehydes to yield triphenylmethane dyes [14]; according to MALOWAN [15] indole derivatives form the following condensation product:



Method

The chromatograms are dried in a stream of cold air, immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution. They are then dried in a stream of cold air.

Alkaloids produce variously colored chromatogram zones (yellow, pink, brown, purple) on a light background [2]. Indole and the catechins appear red [4, 5, 8, 9, 12, 16]. If the catechins are acetylated it is necessary to heat to 105 °C for 5 min after treatment with the reagent [8]. Lysergic acid derivatives should also be heated to 75 °C for 5 min.

Note: The reagent can also be applied by first treating the chromatogram with an unacidified solution of vanillin and then exposing it to hydrochloric acid vapor [3, 9]. Catechin derivatives should be evaluated rapidly (within 10 min), since the red coloration is not stable in daylight and fades relatively quickly [5, 9].

The detection limits in substance per chromatogram zone are 500 ng for alkaloid [2], for indole or ergot alkaloids they are as low as 10 to 25 ng per spot [16].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, NH₂ Diol, CN, RP, cellulose and polyamide layers.

Preparation of the Reagent

Dipping solution	Dissolve 1 g vanillin in 70 ml ethanol (96%) and cautiously add 10 ml fuming hydrochloric acid (37%).
Spray solution	Dissolve 1 to 5 g vanillin in 100 ml hydrochloric acid (37%) [1, 7, 12, 13] or 50 percent methanolic hydrochloric acid [2].
Storage	The reagents are stable for longer periods.
Substances	Vanillin Hydrochloric acid, fuming (37%) Ethanol (96%) Methanol

Procedure Tested

Indole Derivatives [16]

Method	Ascending, one-dimensional, 2-fold development in a trough chamber with 5 min intermediate drying in a stream of cold air. The first development was carried out in an unsaturated normal chamber, the second with chamber saturation. Direct light was excluded.
Layer	HPTLC plates Silica gel 60 F ₂₅₄ (MERCK), which were prewashed by immersion overnight in 2-propanol and then dried at 110°C for 20 min before application of the samples.
Mobile phase	1. Dichloromethane – ethanol (99.5%) (50+50). 2. Dichloromethane – <i>n</i> -hexane – ethanol (99.5%) (9+2+1).
Migration distance	1. 1 cm 2. 4 cm
Running time	1. 2 min 2. 5 min

Detection and result: After the second development the chromatogram was immersed in the dipping solution for 1 s and then heated to 70°C for 5 min (hot plate).

Ergobasine (= ergometrine, “ hR_f ” 20–25)*), ergotamine D-tartrate (“ hR_f ” 35–40) and ergotaminine (“ hR_f ” 60–65) produced violet chromatogram zones on a colorless background. Indole (“ hR_f ” 75–80) acquired a strawberry red color even in the cold. The detection limits in substance per chromatogram zone were 8–10 ng (indole) and 20 to 25 ng (ergot alkaloids).

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda_{\max} = 510$ nm for indole and $\lambda_{\max} = 580$ nm for the ergot alkaloids (Fig. 1).

*) The figures given were calculated as hR_f values even though two developments were involved.

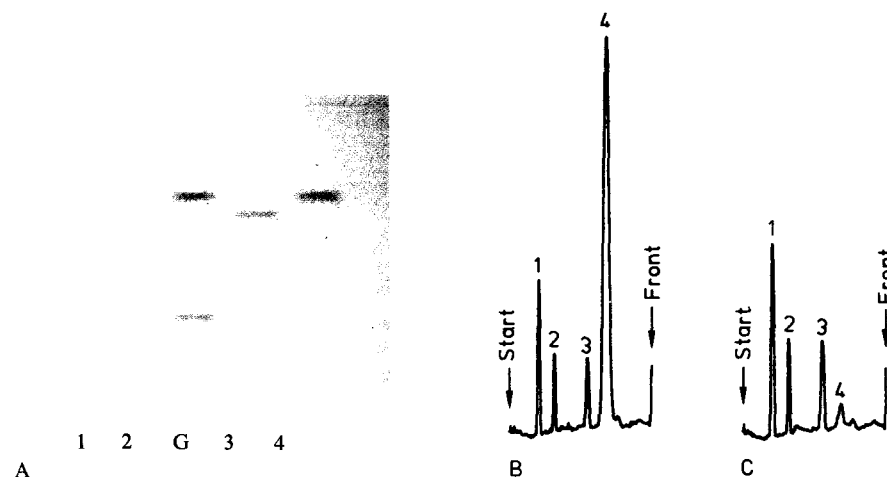


Fig 1: Chromatogram of ergot alkaloids and indole (A) and reflectance scans of chromatogram track G with ca. 80 ng each substance per chromatogram zone measured at $\lambda_{\max(\text{indole})} = 510$ nm (B) and at $\lambda = 580$ nm (C): 1 = ergobasine, 2 = ergotamine, 3 = ergotaminine, 4 = indole.

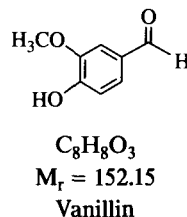
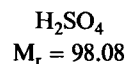
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Vanillin-Sulfuric Acid Reagent

Reagent for:

- Steroids [1-4]
 - e.g. anabolics [5]
 - such as boldenone, testosterone, estradiol
- Bile acid methyl esters [6]
- Essential oil components (4, 7-10)
 - e.g. terpene hydrocarbons (3, 11), farnesol (12)
- Sesquiterpene derivatives [3, 13]
 - e.g. parthenin, hymenin, xanthinine, xanthumine [14]
- Iridoids [15]
- Carotinoids [3]
- Polyacetylenes (polyolefins) [3]
- Phenols [3, 4]
 - e.g. 3-methoxy-4-hydroxyacetophenone [16], holocalin [17]
 - e.g. antioxidants [18]
 - gallate esters, 2,6-di-*tert*-butylphenol
- Catechins, tannins [1]
- Valepotriates [1]
- Flavonoids [3]
- Cardenolides (cardiac glycosides) [1, 19]
- Ginsenosides [20]
- Higher alcohols [4]
- Fatty acids [3]
- Antibiotics
 - e.g. lincomycin [21], primycin [22]
 - monensin, narasin, salinomycin, lasalocid [23]
- UV absorbers [24]
 - e.g. eusolex, cyasorb, prosolal, parsol, witisol



Preparation of the Reagent

Dipping solution	Dissolve 250 mg vanillin in a mixture of 50 ml 1-propanol and 50 ml carbon tetrachloride and cautiously add 2.5 ml conc. sulfuric acid portionwise with stirring and cooling to -15°C [22].
Spray solution	Dissolve 0.5 g vanillin in a mixture of 85 ml methanol, 10 ml acetic acid and 5 ml conc. sulfuric acid that has been prepared under cooling (ice bath) [10]. The literature also contains other compositions, e.g. solutions of 0.5 to 5 g vanillin in 1 to 20% ethanolic [3, 7, 14, 15, 20] or methanolic [18, 21, 23] sulfuric acid, occasionally also in 80 percent ethanolic [4, 6, 25] or undiluted conc. sulfuric acid [4, 5, 11, 12], where the addition of 3 drops acetic acid is recommended in some cases [3, 14].
Storage	The spray solutions should always be made up fresh [3]. The colorless dipping solution can be stored in the deep freeze for 2 weeks [22].
Substances	Vanillin Sulfuric acid (95-97%) Acetic acid (100%) Methanol Carbon tetrachloride 1-Propanol

Reaction

The general aspects of the "aldehyde-acid" reaction were discussed in Chapter 2. Thus it is readily understood that catechins, for example, can react with aromatic aldehyde in the presence of strong acids to yield colored triphenylmethane dyes [26].

METHOD

The chromatograms are dried in a stream of cold air, immersed twice for 1 s (with intermediate drying in a stream of cold air) in the dipping solution [22] or sprayed once homogeneously with the spray solution and then slowly heated, e.g. on a hot plate, to 70°C for ca. 10 min [3] or heated to 100–120°C for 2–15 min [4, 12, 15, 18, 22, 25].

Differently colored chromatogram zones (yellow to dark violet) appear, some before heating, on a light background; the colors alter over a period of 24 h, exceptionally over 48 h (polyolefins) [3]. Monoterpenes steroids and carotinoids yield bright yellow to violet zones [3, 25], fatty acids yield gray colors [3], flavonoids [3], methyl esters of bile acids [6] and lincomycin [21] are colored yellow. The chromatogram zones of farnesol are reddish [12] and primycin gray-violet on a pink-colored background [22]. Cardenolide aglycones, that are not oxidized at the C-14 atom of the steroid skeleton, form blue-colored spots [1].

Note: The chromatogram zones exhibit a broad spectrum of colors [3, 12] that is very dependent on the duration and temperature of heating. Therefore the optimum reaction conditions must be determined empirically. With a few exceptions (ferulic, 4-amino-benzoic and cumarinic acids) aromatic carboxylic acids do not react [3]. The reagent in 80% ethanolic sulfuric acid is reported to be most sensitive for steroids [25].

The detection limits in substance per chromatogram zone are 50–100 ng for sesquiterpene lactones [3] and 100 ng for lincomycin [21] and primycin [22].

It is possible to replace the vanillin in the reagent by 4-dimethylaminobenzaldehyde, 4-hydroxybenzaldehyde, salicylaldehyde, *m*-anisaldehyde, cinnamaldehyde, 4-hydroxybenzoic acid or vanillic acid [3]. However, the range of colors obtained is not so broad.

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000 and RP layers.

Essential Oil Components [10]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F ₂₅₄ (MERCK), that had been prewashed before application of the samples by developing once to the upper edge with chloroform – methanol (50+50) and then drying at 110°C for 30 min.
Mobile phase	Toluene – ethyl acetate (93+7).
Migration distance	13 cm
Running time	30 min

Detection and result: The chromatogram was dried in air (!) and then evaluated under short-wavelength ($\lambda = 254$ nm, Fig. 1A) and under long-wavelength ($\lambda = 365$ nm, Fig. 1B) UV light and documented photographically [27]. It was then sprayed homogeneously with the reagent solution and observed as it was heated to 120°C on a hot plate. Chromatogram zones of various colors (Fig. 1C, see Table 1 for substance assignments) are produced whose color shades and intensities alter with increasing duration and temperature of heating. Hence the optimum duration and temperature of heating must be determined empirically.

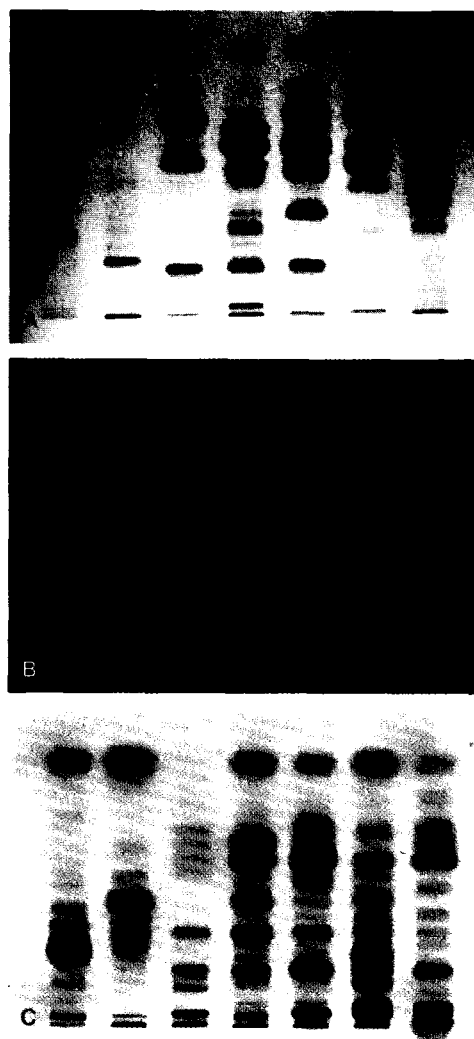


Fig 1: Chromatograms of various essential oils photographed (A) under short-wavelength ($\lambda = 254$ nm) and (B) long-wavelength ($\lambda = 365$ nm) UV light before treatment with the reagent and (C) in daylight after detection with the vanillin – sulfuric acid reagent.

Table 1: Essential oil components with hR_f value, color and detection limits.

Substance	Color on det.	hR_f value	Detection limit
Anethole	brown	55–60	500 ng
Bergamot oil (2 major zones)	reddish brown	20–25	
	reddish brown	45–50	
Bornyl isovalerate	dark blue	75–80	100 ng
Cineole	dark brown	30–35	
Hydroxycitronellal	dark brown	5–10	
Isobornyl acetate	brownish gray	55–60	200 ng
Isomenthone	yellowish green	35–40	
Lavender oil (3 major zones)	bluish gray	20–25	
	dark blue	45–50	500 ng
	pink	65–70	
Menthol	dark blue	15–20	
Menthone	yellowish green	45–50	200 ng
Menthyl acetate	green	45–50	
Patchouli oil (2 main zones)	violet	30–35	
	violet	70–75	500 ng
Pulegone	dark brown	30–35	
Rose oil	brownish gray	10–15	
Rose oxide	brownish gray	35–40	200 ng
Sandalwood oil (2 major zones)	grayish blue	20–25	
	grayish blue	70–75	
Thymol	strawberry red	40–45	200 ng

Note: In order to document the color changes photometrically during the heating process the hot-plate was placed on the document table of a reproduction camera placed in the fume cupboard (!). The camera was focused when the plate was cold and the heating process was commenced. When the first colored chromatogram zones appeared they were photographed and photographs were then taken at regular intervals, e. g. every 10 s, until the colors on the chromatogram ceased to change.

In situ quantitation The fact that the colors of the chromatogram zones changed means that quantitative in situ evaluation is only meaningful in rare cases.

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